JOURNAL OF THE
NATIONAL CANCER INSTITUTE

VOLUME 4 *October 1943* NUMBER 2

Production of Malignancy in Vitro. I. Method of Cleaning Glassware

By WILTON R. EARLE, *senior cytologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

During a series of investigations of the action of methylcholanthrene on tissue cells cultivated in vitro, the problem arose of how to clean satisfactorily the glassware used. Inasmuch as methylcholanthrene is apparently active in extremely slight traces, it was feared that the usual cleaning with soap, trisodium phosphate, or other detergents could not be relied upon to remove the agent. The use of dichromate-sulfuric acid cleaning solution was ruled out because of the unsatisfactory and erratic growth of cultures in glass containers cleaned by this method, possibly owing to the adsorption of chromium salts by the glassware.[1]

The method and apparatus finally developed [2] for cleaning glassware with hot sulfuric acid with the admixture of a little nitric acid have proved quite satisfactory. The method seems to be particularly suitable for cleaning glassware used in biologic work where traces of certain adsorbed material like chromium must be eliminated and where common detergents are unsatisfactory. The apparatus is shown in figure 1.

A steam-jacketed duriron kettle with a capacity of about 13 gallons is used.[3] The kettle, which is equipped with pressure gage, steam-pressure reducer, condensate outlet valve, and other fittings, operates at a steam pressure of 15 to 18 pounds.

The cleaning fluid reservoir (fig. 1) consists of four 20-l. pyrex bottles, each with its rubber stopper wired in. Each bottle is encased in a sheet-metal shield with soldered seams, which lessens the chance of breakage through an accidental blow and at the same time serves to reduce the danger from acid in case of failure of any unit of the glass reservoir. The bottles are connected with one another and with the kettle by glass tubing of 15-mm. outside diameter. The stopcock closing

[1] *Laug, E. P.: Retention of dichromate by glassware. Indust. & Engin. Chem., Analyt. Ed., 6: 111–112 (1934).*

[2] *Dr. B. B. Westfall, of the Division of Chemotherapy, National Institute of Health, made some preliminary determinations on the rate of loss of water from hot sulfuric acid, and it was on the basis of these determinations that the method was finally worked out. Dr. Jonathan L. Hartwell, of the National Cancer Institute, also made suggestions in regard to developing the method. The glass acid line was constructed by E. L. Schilling.*

[3] *The kettle is manufactured by the Duriron Co., Dayton, Ohio, and can be furnished in a larger size. According to the Handbook of Chemistry and Physics, 23d edition, duriron, a resistant alloy, is composed of iron, 84.3 percent; silicon, 14.5 percent; carbon, 0.85 percent; and manganese, 0.35 percent.*

548963—43——1

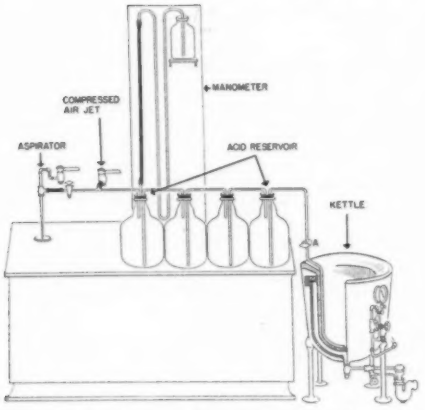

FIGURE 1.—Diagram of apparatus used for cleaning glassware with hot sulfuric and nitric acids. The sheet-metal shields around the acid reservoir bottles and the canopy over the kettle are omitted in the figure.

the acid line from the reservoir to the kettle is of 10-mm. bore through the plug. The glass inlet and outlet tubes of each bottle extend well below the rubber stopper so that the acid never comes in contact with the stopper. The glass tubing into the kettle is so shaped that it conforms to the curvature of the wall of the kettle which thus supports it.

The manometer indicates the amount of pressure or vacuum in the bottles. The fluid used in it is 80 percent sulfuric acid. Mercury should not be used because of the hazard of contaminating the acid. The manometer also serves as a safety release to limit the pressure or suction that may be exerted on the glass reservoir.

Fragile glassware, glassware that must be heated slowly, or very heavy glassware should preferably be put in the kettle as the acid runs in from the reservoir. Lighter units or less fragile glassware may be put into a kettleful of hot acid. As with any hot acid solution, care must be taken to add nothing that is likely to form an explosive mixture. While water is never poured into the hot acid, frequently glassware that is either wet or contains 1 to 2 cc. of water is placed in the hot acid bath. This has not proved hazardous.

In heating the kettle, steam is first admitted slowly through the needle valve, and only after the kettle is thoroughly heated is the main steam inlet turned on. In our work, where it is essential to destroy traces of carcinogens on the glassware, the heat is usually left on 1 day after the specific gravity of the acid indicates a concentration of 80 percent or higher. The steam is then turned off and the kettle allowed to cool. When reasonably cool, the acid is returned to the reservoir and the glassware lifted out and rinsed.

In removing the glassware from the kettle, the operator should wear rubber gloves and a rubber apron. Thin surgical gloves have been found to be better than the more cumbersome heavy acid cleaning gloves customarily used.

Two kettles, each with its own reservoir, are used alternately so that one kettleful of clean glassware is ready for rinsing at the middle, the other at the end of the week.

The cleaning solution leaves no residue from the oxidizing agent and can be used as long as desired, with the addition of sufficient sulfuric acid to make up what is lost, and a little nitric acid at each run. In our unit, the original acid is still in use after nearly 2 years and has received only enough fresh sulfuric and nitric acids to make up loss of volume. About 100 cc. of the latter is added per week.

Glassware cleaned in this way and then well-rinsed in distilled water has now been used for long-term tissue cultures for over 2 years and has shown no injurious action on the living cells. The results are far superior to those formerly obtained when glassware was cleaned in dichromate-sulfuric acid.

By means of the water aspirator any fluid in the kettle may be drawn over into the cleaning fluid reservoir; when it is desired to force the fluid back into the kettle, a low-pressure source of compressed air connected with the last bottle in the series is used. In our work, the source of compressed air is the laboratory air-pressure line.

The cleaning solution is reagent-quality concentrated sulfuric acid diluted to approximately 80 percent with distilled water. To each kettleful of sulfuric acid about 100 cc. of concentrated reagent-quality nitric acid is added, and the whole is mixed by drawing the fluid into the reservoir and then discharging it into the kettle. When the mixture is allowed to stand in contact with air, it absorbs some water, but on being heated at 15 to 18 pounds pressure it loses water rapidly and reaches an acid concentration of about 82 percent, as determined with a hydrometer. When heated at this steam pressure, the fuming from the solution is slight, even after the addition of more nitric acid. A light sheet-metal canopy, painted with asphaltum and fitted with a small exhaust fan, is sufficient to handle the fumes.

In the early trials, concentrated sulfuric acid was used without dilution. However, heavy fuming resulted when the undiluted acid was heated, and when cool, the acid absorbed water from the air until the total volume was in excess of the capacity of the kettle. The use of 80 percent sulfuric acid is therefore advised. In some of the tests, 30 percent hydrogen peroxide was used instead of nitric acid. As an oxidizing agent, it was satisfactory, but had the serious objection that the liberation of minute bubbles of oxygen from the solution resulted in the spraying of tiny droplets of acid into the air. Its use was discontinued. The use of a lid on the kettle was likewise discontinued inasmuch as the tests showed that the lid slowed up the loss of water from the cleaning solution and consequently interfered with its action.

In other tests, steam at different pressures was used; a pressure of 9 to 14 pounds gave substantially slower cleaning action than did the pressure of 15 to 18 pounds finally adopted, while a pressure of more than 18 pounds resulted in a decided increase of fuming from the acid bath.

The efficacy of this acid bath in the destruction of methylcholanthrene was tested by taking small tubes containing 1-mg. lots of crystalline methylcholanthrene and 1-mg. lots of crystalline methylcholanthrene plus a little serum and subjecting them to the action of the various cleaning mixtures considered for use in the bath. It was found that with either 80 percent sulfuric acid plus the addition of a little 30 percent hydrogen peroxide or 80 percent acid plus a little nitric acid, charring occurred within a very few minutes at room temperature, with complete solution and clearing of the material on gentle warming.

In cleaning glassware for biologic work, it should first be rid of any heavy metal salts before being placed in the kettle of acid, as an admixture of heavy metal salts would leave a residue in the acid that might possibly contaminate glassware cleaned in the solution later.

Production of Malignancy in Vitro. II. Photomicrographic Equipment

By Wilton R. Earle, *senior cytologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

In this laboratory a great deal of trouble has been experienced in obtaining routine photographic records of tissue cultures. While the usual commercial photomicrographic apparatus has been used for occasional photographs, when routine photographs of long series of living cultures held at 38° C. are desired the apparatus available has not been satisfactory. Often there has been too little assurance, until the negative has been processed, that a satisfactory record has been obtained.

The general requirements for a photomicrographic unit for our work are as follows:

(1) In addition to photographing the usual histologic sections, it is essential that the apparatus be designed to take photographs of cultures of living tissue cells in Carrel flasks, on hollow ground slides, or in similar culture dishes, with the cells in their usual position under a layer of culture fluid. To do this, it is necessary to use an inverted microscope mounted vertically above the camera.

(2) In order to use a wide range of magnifications with large plate sizes, it is essential that the camera have a bellows extension of at least 48 inches. To have this long extension and at the same time retain easy access to the microscope, a horizontal camera is required. Since the optical axis of the camera is thus at right angles to that of the microscope, the image from the microscope must be reflected into the camera.

(3) Cultures under examination must be held at 38° C. during the photographic process. The whole microscope must therefore be enclosed in a 38° air bath, with adequate room in the bath for several racks of cultures. To maintain even heating, a thermoregulator and air circulation of the bath are essential.

(4) Since nearly all high-intensity microscope light sources are designed to burn with their optical axis in or near the horizontal, it is necessary to keep the axis of the light source horizontal and to reflect the light into the substage with the substage mirror.

(5) Adequate sources of light are necessary for both low and high magnifications, with the light for the low magnifications, at least, under accurate intensity control while the image is under examination on the ground glass.

(6) The range of objectives extends from a 100-mm. photographic objective down to the usual oil-immersion objective. The substage must handle very low power condensers.

(7) The camera must take different standard sizes of plates up to 8 x 10 inches.

(8) The whole assemblage should be mounted as a rigid unit in order to be free from vibration not only within itself but also from the floor. Construction must therefore be sufficiently heavy to damp out vibrations.

(9) Maximum ease and rapidity of operation are essential. It is therefore necessary to extend the controls out to where they can be conveniently manipulated while the image on the ground glass is being examined.

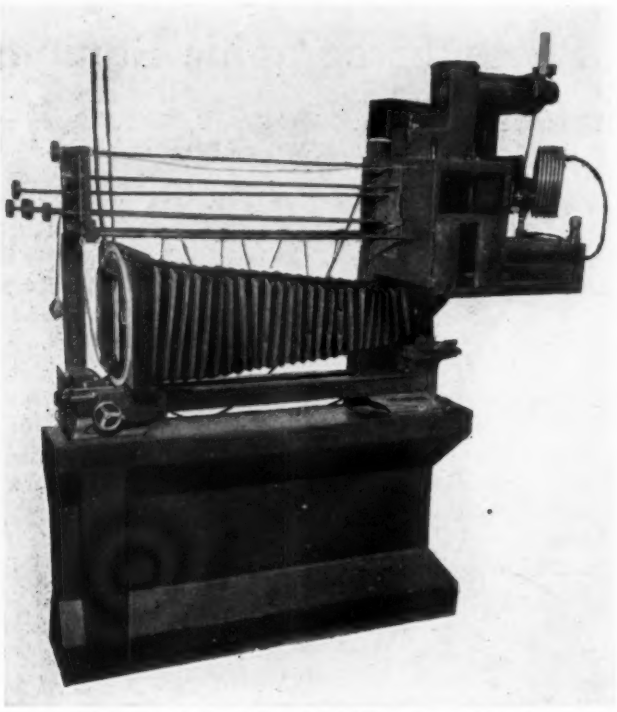

FIGURE 1.—General view of the photomicrographic unit.

(10) Provision must be made for accurate determination of the correct exposure time. Owing to widely divergent optical properties of different cultures, the means used must depend not on any system of arbitrary setting of the optics involved but on an actual measurement of the light reaching the ground glass.

APPARATUS

An attempt was first made to modify some model of photomicrographic apparatus obtained commercially. For the types examined, the modifications proved to be extensive and even with them the apparatus fell far short of what was wanted. It was therefore decided to build the unit desired. Commercial parts were employed wherever their use simplified the construction. Figures 1, 2, and 3 are views of the photomicrographic unit constructed.

The apparatus has now been in use for over a year. While it includes a number of specialized features unnecessary for general photomicrographic work, it has been found very satisfactory for the work for which it was designed, and a number of its features would probably be of great advantage in more general photomicrography. In the following description although the exact details of construction are omitted, the general design and construction[1] are outlined.

[1] *Much of the mechanical design of the equipment was worked out by Dr. F. S. Brackett, formerly of this Institute. Other parts were designed and the whole unit was constructed under the supervision of Laurence R. Crisp, with the collaboration of the personnel of the various shops of the National Institute of Health.*

Microscope Stand

No satisfactory model of inverted microscope could be found. The stand used (current model No. 5 Spencer stand, complete with circular graduated mechanical stage) was selected because it is simple and rigid; the substage and mechanical stage heads are conveniently located and fixed in position; the tension on the fine adjustments can be tightened by means of an adjustable screw head so that the microscope can be satisfactorily used in an inverted position. The microscope was ordered with the following special alterations: (1) Substage pinion and stage adjustment heads operable from both sides of the microscope; (2) the substage rack lengthened to 6 inches; and (3) inclusion of an extra body slide-body tube assembly.

A further alteration was made in our shops by inverting the stage. The stage was first removed; a metal saddle was made and fitted around the arm of the frame; the stage, in an inverted position, was bolted to the saddle and mounted so that the flat stage surface on which the object under examination would rest was at the same level on the frame as it had been originally. The machine work involved was probably the most exacting step in the whole construction, as great care was necessary to relevel the stage exactly. The general design of this altered stand is shown in figures 4 and 5.

To hold a Carrel flask on this type of inverted stage a special flask holder was made. The holder is shown in figure 6.

Base

The base (figs. 1 and 2) consists of a concrete horizontal bench, from the rear of which near one end is a pillar. The bench supports the horizontal camera, while the pillar supports the microscope and its accessories. Concrete was used for the base because of its cheapness, its relative ease in handling, its great rigidity, and vibration-absorption properties. The complete base and pillar were cast in situ as one unit in a plywood form. The concrete was liberally reinforced in all three axes with ½-inch deformed steel rods. After seasoning, finishing, and drying out, the concrete mass was painted.

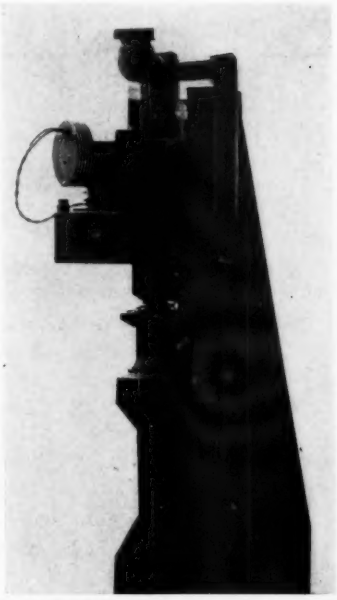

Figure 2.—Photomicrographic unit, seen from the microscope end. Note the arrangement of the microscope lamps and air circulation ducts.

The whole base is supported and insulated from the floor by three pads of Vibracork 2 inches thick, so placed as to carry the mass on a stable three-point support. The area of these pads was calculated so that the load on each is approximately that recommended by the manufacturers as being optimal for vibration absorption.

Microscope Bed Plate

A steel plate ½ x 14 x 16 inches long is securely fastened to the face of the pillar

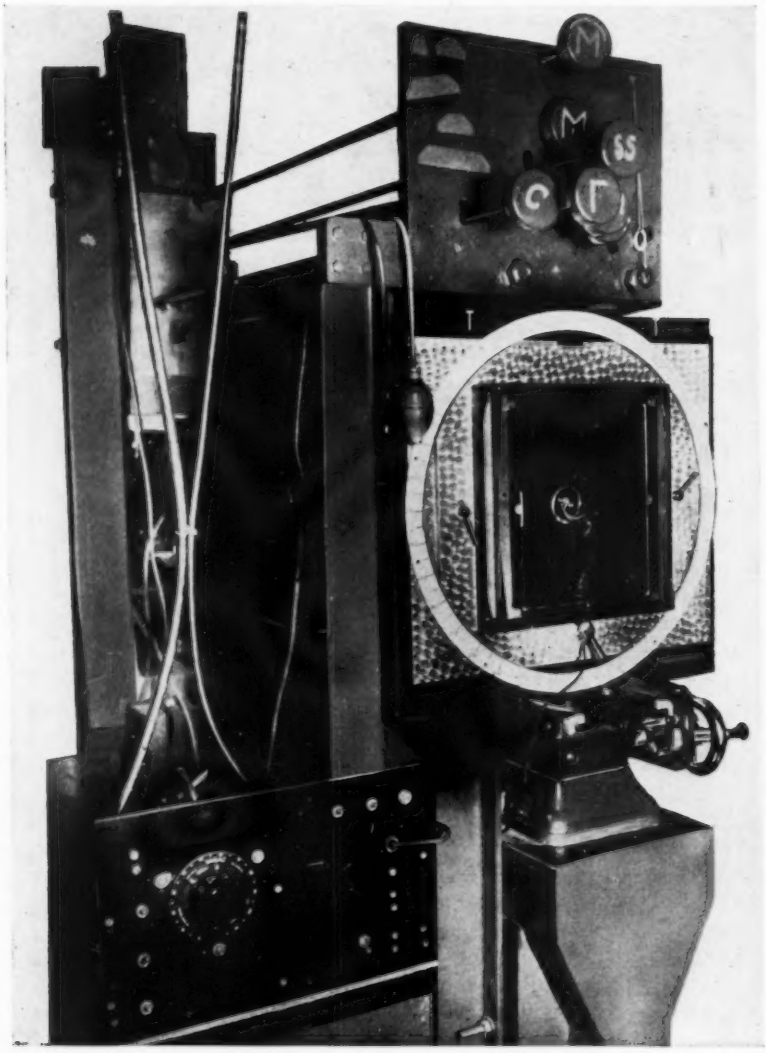

FIGURE 3.—Rear end of the camera unit. Note the electrical control panel, the extended control handles, and the rotary plateholder. The photoelectric cell on its support may be seen through the ruled focusing glass.

near its top by four bolts inserted through preformed holes in the pillar. The base of the microscope is securely bolted to this plate.

AIR BATH HOUSING, AIR HEATING, AND CIRCULATION

The general shape of the air bath housing is shown in figures 1, 2, and 4. The

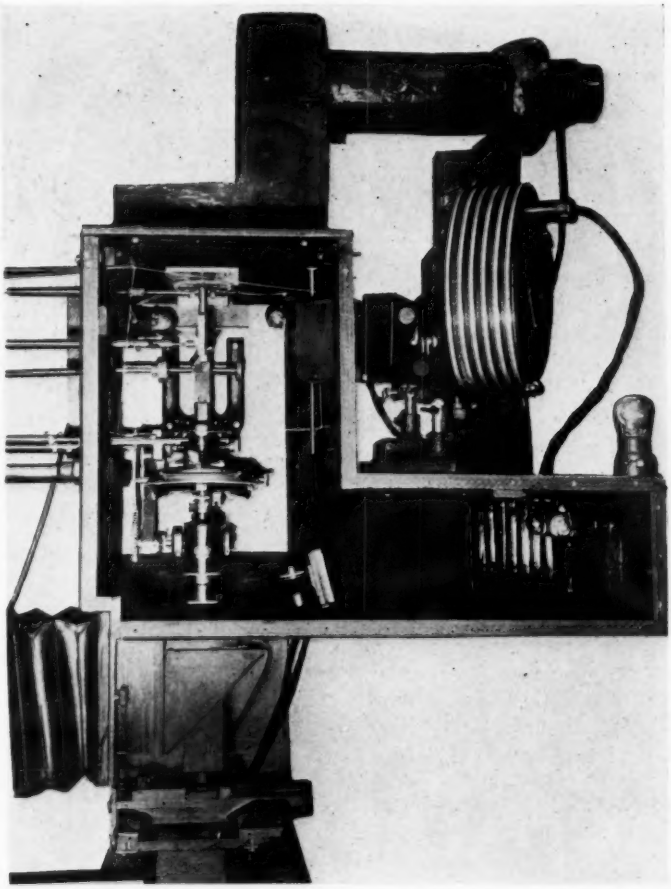

FIGURE 4.—Close-up view of the front of the air-bath unit (with front removed) and mirror housing. The shutter is on top of the mirror housing.

housing is made of ¾-inch plywood; double glass windows are inserted in its front. Hand holes sealed with flaps of rubber sheeting 2 mm. thick are provided through which cultures may be manipulated. Racks of cultures can be inserted or removed through the small door (fig. 2) at one end of the housing. The housing is suspended from the heavy steel base on which the microscope is bolted.

Two holes each 2 inches in diameter open from the top of the housing into an insulated air duct which runs up to a small blower unit suspended from the ceiling of the room. From thence a duct is run down to the extreme diagonal corner of the air bath and opens into a small extension of the latter (fig. 2). In this extension are four 200-watt projection bulbs covered with aluminum foil, which are used to heat the air bath. They are wired in pairs in parallel series to minimize the hazard of their burning out when in constant use. A series of deflecting louvers in front of

548963—43——2

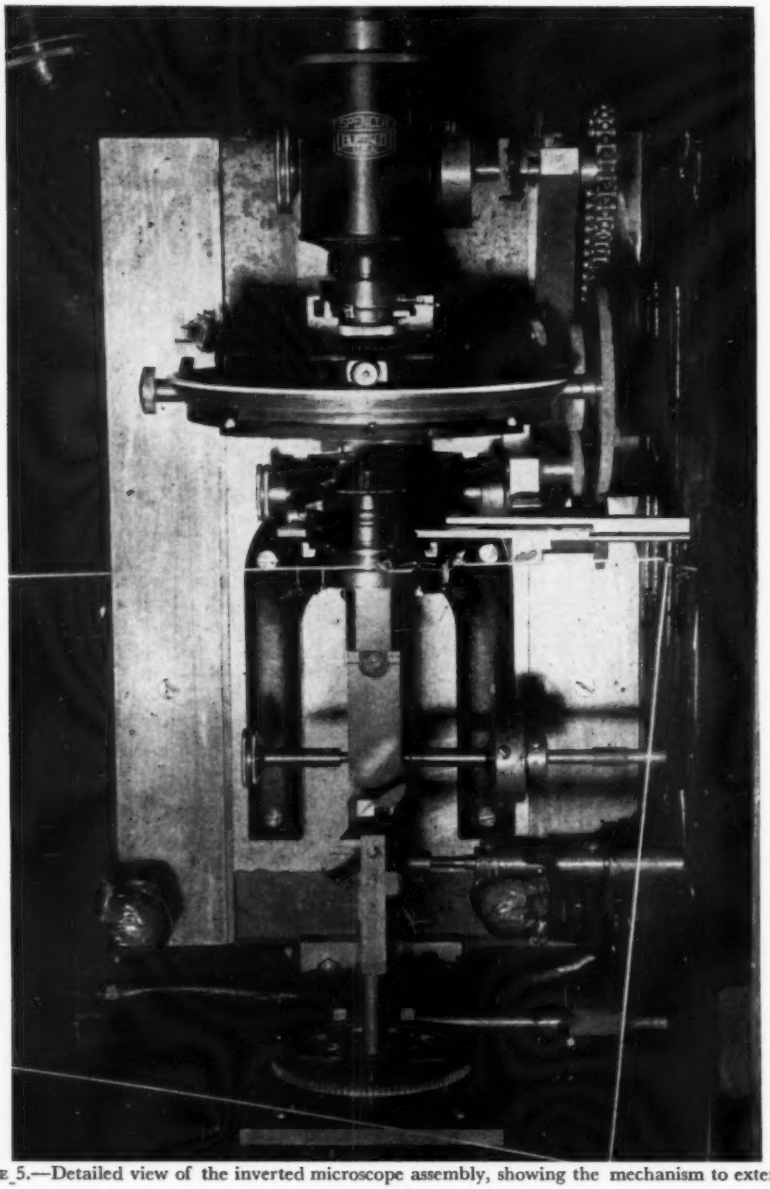

FIGURE 5.—Detailed view of the inverted microscope assembly, showing the mechanism to extend the controls.

the bulbs (fig. 4) deflects the air flow so that the whole area of the bath is held to a temperature of 38.0° to 38.5° C.

The air circulator (figs. 2 and 4) hangs from the ceiling. Its inlet and outlet are brought to within one-half inch of the open ends of the air ducts of the bath, and the ends of the ducts are then connected with the inlet and outlet of the air bath by wrapping with a single layer of thin latex rubber dam and over this one layer of light, soft cloth. This type of connection keeps any vibration of the air circulator from shaking the photomicrographic unit.

The thermoregulator is of the enclosed mercury-in-glass type, which we have found can be relied upon to hold its setting over a number of years.

Substage Mirror

The substage mirror is removed from the microscope frame and suspended in a yoke. The yoke is suspended from the shaft of a worm gear about 3 inches in diameter (fig. 5). The gear, together with the worm actuating it, is supported by a bearing plate mounted from the top of the air bath directly above the miscroscope. The extension of the horizontal shaft on which the worm is mounted is run out through the side of the air bath. The operation of the shaft rotates the mirror about a vertical axis. The circular mirror is suspended in its yoke by two diametrically opposed pivots so that the slope of the mirror can be changed at will. The angle of the slope is regulated by a tight-fitting, finely threaded screw which works through a threaded block mounted on the mirror yoke, and against the top upper half of the back of the mirror. The mirror is held firmly against the end of the screw by a small coil spring, one end of which is attached to a small hook on the upper edge of the mirror and the other end to the block through which the mirror-adjusting screw is threaded. The handle extension of this screw is run out through the side of the air-bath housing.

Figure 6.—Flask holder, to carry D 3.5 Carrel flasks in the mechanical stage of the microscope. The spring clips, 0.25 mm. thickness, are made of phosphor-bronze.

Illuminating System

An incandescent ribbon filament lamp is satisfactory for all photomicrographic work with relatively low magnifications, since with this type of illumination the field is evenly lighted and the intensity can be easily controlled by a variable transformer of the Variac type in circuit with the lamp. At extreme magnifications, however, the lamp should be supplemented with one of the enclosed-arc type. Figure 2 shows both of these lamps, mounted alongside each other on the horizontal shelf formed by the air bath. Care must be taken to adjust the optical axis of each lamp to the horizontal and to set the two optical axes at the same height; but once adjusted, a shift of the angle of the mirror about the vertical axis centers the light from either lamp on the substage condenser.

Camera Bed and Mounting, and Mirror Housing

The general design of the camera is shown in figures 1 and 3. The bed of the camera consists of the 54-inch bed of a 10-inch metal cutting lathe [2] which was purchased complete with two carriages and rack, but without headstock and tailstock assemblies and without screw-cutting worm. Both carriages are equipped with

[2] *Manufactured by the Atlas Press Co., Kalamazoo, Mich.*

the cross slide of the tool rest but without the compound tool rest. One carriage was obtained with rack and with hand wheel and gear for working on rack, and the other without complete apron assembly. The complete lathe, or camera bed, is on the horizontal shelf of the concrete base to which it is fastened by four bolts embedded in cups in the concrete base. An iron casting, the general shape of which is shown in figures 1, 2, and 4, is bolted to the front end of the lathe bed (end nearest the microscope). The lathe carriage with no apron assemblage is mounted on this iron casting, the slides of the carriage being transverse to the axis of the lathe; the carriage is then locked in this position.

The camera mirror housing can be removed for cleaning or adjustment by moving it along the transverse slide of the carriage, or it can be moved along the slide and then rotated on its vertical axis so that the mirror can easily be reached for cleaning. The mirror is made of heavy glass, approximately 4 x 6 inches in size, aluminized on the front surface. It is mounted at 45° to the optical axes. The aperture on the microscope side of the housing through which light reaches the camera mirror from the microscope is circular and is 3 inches in diameter. The aperture on the camera side of the housing is square, 4 x 4 inches in size. These holes are as large as possible in order not to reduce the angle of the cone of light from the microscope. On top of the camera mirror housing and closing the circular aperture is a Packard Ideal photographic shutter (fig. 4) with a 3-inch opening. The rubber tubing and bulb by which the shutter is operated are run out to the ground glass end of the camera.

The front end of the camera is rigidly mounted on the lathe carriage by a vertical steel standard bolted thereto (fig. 4). The whole plateholder (rear) end of the camera is rigidly supported on the second lathe carriage. With the hand wheel actuating the lathe carriage rack and pinion movement, the carriage can be moved along the length of the bed, and with the lock it can be securely fastened at any extension along the bed (fig. 1). The bed is graduated in millimeters so that any desired camera extension can be read and accurately reproduced.

Bellows and Bellows Mounting

The bellows allows a maximum extension of 50 inches. At the front of the camera it is 8 inches square, while at the plateholder end it is 14 inches square (free inside size). A 14-inch square is sufficiently large to allow the complete rotation of an 8 x 10 inch plateholder without having the bellows cut the light reaching the corners of the plate.

Rotary Plateholder

To align the image on the ground glass easily at any angle, the back of the camera is so designed that the ground glass and plateholder are rotary (fig. 3). The rear surface of the rear bellows support is closed by a metal plate $\frac{5}{16}$ inch thick, held in place by knurled head set screws. Through the center of this plate is a hole $14\frac{1}{2}$ inches in diameter, covered on the outside by a circular plate $17\frac{1}{2}$ inches in diameter and $\frac{5}{16}$ inch thick. This plate has a $10\frac{1}{2}$-inch square hole through the center and is mounted to the metal back of the camera by a series of clamps which reach in around the edges of the 14-inch hole in the back plate of the camera and hold the circular plate to the rear surface of the plate first described and yet allow it to rotate through an angle of 360°. Two of these clamps, on opposite sides, carry locking devices so that the rotary metal plate can be locked at any angle of

rotation. The angle can be determined or set by a graduated circle mounted on the first plate, and a pointer mounted on the outer side of the rotary plate.

The rotary metal plate of this unit has a mount fitted on its outer side which takes the whole viewing back of a standard 8 x 10-inch-view camera. A series of regular commercial plateholder backs are used with this mount, taking plates 8 x 10, 5 x 7, 4 x 5, or 3¼ x 4¼ inches in size, thus permitting rapid interchange of plateholders of different sizes. Each back carries its own ground glass of correct size for focusing that size plate.

Extension of Controls

To support the bellows and the extension rods of the controls, near the rear end of the camera a heavy angle-iron support is bolted onto the side of the concrete base and run up alongside the camera so that it just clears the camera and extends out over the camera body (figs. 1, 3). On this support a wooden panel, mounted over the camera, is tied by two ¾-inch steel rods to the side of the air bath facing the back of the camera. The bellows is supported, at four points along its length, from metal hooks sliding along these rods. The wooden panel supports the extended control rods which actuate the different controls of the microscope. These rods consist of thin-walled metal tubing ending in handles of convenient size and shape (figs. 1 and 3).

The extension of the various microscope controls offered a number of minor problems. To open and close the iris, a heavy linen thread is rigged to run through hooks on the inner walls of the air bath, out through a hole in its side, and through a hole in the standard over the camera, and terminates in rings of convenient size, white and black, to indicate opening and closing of the iris, respectively (figs. 3, 4, and 5).

The shaft controlling the rotation of the angle of the mirror about the vertical axis (fig. 5) runs out through the side of the air bath, where a universal coupling of the Curtis type connects it with a light-weight brass tube that extends out through the standard over the camera and ends in a handle. The control for regulating the angle of incline of the mirror is similarly arranged, as are the extension from the fine adjustment head of the microscope and the shaft for raising and lowering the substage (fig. 5).

Some difficulty was experienced in running out the extensions for the coarse adjustment and for the mechanical stage. The coarse adjustment varies slightly in position on the microscope as the fine adjustment shifts; it was so low in the inverted microscope frame that if extended in a straight line it would not have cleared the back of the camera. A sprocket was therefore attached to the coarse-adjustment head by a flexible coupling, which allows the fine adjustment to work through its range unhampered (fig. 5). The sprocket is linked to another sprocket mounted from a support on the microscope bed plate and at such a height from the camera base that the shaft of the second sprocket clears the top of the camera. The extension of the shaft of the second sprocket is carried out of the air bath by a flexible coupling and extension tube, as was done with the mirror control. The sprockets have a ratio of 2:1.

The extension of the mechanical stage controls along their original axes would also have interfered with the back of the camera. The line of extension of the control rods had to be lifted a short distance to clear the back of the camera. To do this, the knurled head of each stage movement shaft was removed, and in its stead

a light brass wheel several inches in diameter was substituted (fig. 5). Each brass wheel has a corrugated rim 3/16 inch wide, and each is actuated by friction with the rubber friction rim of a smaller wheel supported by a pillar from the microscope base plate. The shaft of each rubber-rimmed wheel is extended outside the air bath by universal joints of the Curtis type.

With these various mechanisms, once the object is placed on the stage of the microscope, all adjustments of the microscope can be made from the rear of the camera while holding the image under examination in the ground glass.

Electrical Controls

All electrical controls for the lights are also carried out to a panel alongside the rear of the camera and within easy reach while the operator is viewing the object on the ground glass (fig. 3). The controls include (1) a master radio-type switch opening or closing all circuits, (2) a heavy-duty radio-type switch for turning the enclosed arc lamp on or off, and (3) the circuit for the ribbon filament lamp. This circuit includes a radio-type switch and a Variac type transformer which has a range from complete extinction to full brilliance of the lamp.

Photoelectric Exposure Meter

To control the exposure time, a modified form of the photoelectric-cell unit previously described is used. A Photovolt[3] glass-protected, bakelite-encased photoelectric cell, 1⅛ inches in diameter, is mounted just in front of the ground glass of the camera (fig. 3) and is supported by a metal arm controlled by a handle extending from the back of the camera. In this way the cell can be swung to the center of the field; and after a reading is taken, it can be swung entirely clear of the area of the photograph. The cell is connected by a light, flexible, electric line with a Leeds & Northrup 2500A galvanometer suspended from the wall of one side of the room, and illuminated by an enclosed galvanometer lamp so that a ray of light is reflected from the galvanometer mirror to a graduated scale on the wall opposite. The scale is so placed that it is in front of the operator while viewing the object on the ground glass and while adjusting the variable transformer of the light source or the substage condenser. As the author previously pointed out (*1*), such a photoelectric meter allows a determination of the light intensity reaching the plate through any selected area of the object being photographed. This eliminates any corrections for bellows length, objective, ocular, or other alterations in the optical system, with the sole exception that a correction is needed if a color filter is used, or for stained slides, or other brightly colored objects.

The switch for the galvanometer lamp and the switch in the galvanometer circuit are also mounted on the electric control panel.

DISCUSSION

Many, if not the majority, of the difficulties encountered in the construction of this apparatus arose from the necessity of using the inverted microscope system, which in turn was necessitated by the construction of apparatus to handle living tissue cultures. Where the inverted microscope is not necessary, it is more satisfactory to have an entirely horizontal photomicrographic unit. Whichever one is employed, the mounting of the whole apparatus as a unit on a heavy base insulated from the floor and the use of a heavy lathe type bed for the camera unit give a stability that greatly facilitates operation.

[3] *Manufactured by the Photovolt Corporation, 95 Madison Ave., New York.*

The use of a long bellows, the extension of the controls out to the ground glass, different-size interchangeable plateholders rotary through 360°, easily interchangeable sources of light, the control of light intensity, and the incorporation of a satisfactory photoelectric exposure meter are all features which could profitably be incorporated into the heavy duty photomicrographic equipment.

Particularly in the extension of the controls out to the ground glass, the exact devices will vary greatly with the design of the apparatus. In a number of instances, e. g., the chain-and-sprocket control of the coarse adjustment, the means used to effect the various controls have been relatively crude. Even with such defects, the time saved and the ease and accuracy with which the object can be regulated while under observation on the ground glass have more than compensated for the shortcomings of the mechanism.

When an entirely horizontal photomicrographic unit is used, a number of the devices can be much simplified. For instance, the stage controls and the focusing and substage controls can all be accomplished by the simpler and possibly more satisfactory control used on our microcinematographic equipment (*2*).

One point should be emphasized with regard to the photoelectric cell used. While the use of a photoelectric cell of large diameter increases the sensitivity of the photoelectric readings, the diameter has been kept to a practical minimum. The reason is that frequently in microscopic preparations it is necessary to get correct photographic exposure in a very dense area of limited size, surrounded by one of high light transmission, as for example, a small, very dense culture surrounded by clear plasma clot. With a small photoelectric cell, the surface of the cell may be entirely covered by the image of the dense area, and a correct reading of the light can easily be obtained. The active surface of a large cell could not be correctly covered by the image of such an object, with the result that light flooding into the cell in an uncovered area could easily give such an erroneous value that the photographic record would be ruined.

REFERENCES

(*1*) EARLE, W. R.: A simple photoelectric exposure meter for use in photography. J. Biol. Photographic Assoc., 7: 54–58 (1938).

(*2*) EARLE, W. R., and CRISP, L. R.: Production of malignancy in vitro. III. Microcinematographic equipment. J. Nat. Cancer Inst., 4: 147-164 (1943).

Production of Malignancy in Vitro. III. Microcinematographic Equipment

By WILTON R. EARLE, *senior cytologist, National Cancer Institute,* and LAURENCE R. CRISP, *associate mechanical engineer, National Institute of Health, United States Public Health Service*

INTRODUCTION

In a study of the action of methylcholanthrene on living fibroblasts in vitro, it became necessary to examine such phenomena as rate and manner of cell cleavage and migration. The only practical way was to study them by means of microcinematographic records, but the procedure was complicated by the fact that to eliminate the influence of variation in temperature, age, culture medium, etc., all studies had to be made using the behavior of normal cells as a control. This necessitated simultaneous microcinematographic records of normal and treated cells. A comparison or double microcinematograph was therefore devised.[1] Since the equipment includes a number of features which the authors have not seen described and which contribute materially to its usefulness and ease of operation, it is described in the present paper.

The equipment was designed to meet the following requirements:

(1) To obtain the greatest possible detail, the apparatus is constructed to handle 35-mm. film.

(2) Since the microcinematograph is used primarily in the study of carcinogen-treated cells with untreated cells as controls, it is necessary to have paired optical systems, lights, lenses, and cameras so that two cultures can be photographed simultaneously under identical conditions. This necessitates complications in the mechanical design.

(3) To hold the cultures at 38.0° C., the two microscopes are enclosed in a constant temperature bath sufficiently large to hold two racks of cultures also. Heater, thermostat, and air circulator are essential to insure constant temperature over an extended interval, and a written record of the temperature during the interval of any one film necessitates a thermograph.

(4) Photographs must be made of tissue cultures in both slide preparations and Carrel flasks. Since each culture must be kept in its normal position with the cells under a layer of culture fluid during the process, it is necessary to have each microscope of the inverted type, supported over its own camera. As the cameras and their equipment have a heavy power drive, it was decided to hold the cameras at constant height to avoid mechanical difficulties. The whole microscope-air bath-lamp assembly could then be supported above them from two parallel vertical lathe beds and adjustable to a variable height, while the cameras were carried below them on a third lathe bed, this one horizontal.

(5) The exposure frequency must range from one frame per second to one frame each 2 minutes. The range limits are held to the greatest that would probably ever be needed. Frequencies greater than one per second were therefore purposely excluded as this rate threatened trouble from vibration and entailed substantial additional cost.

[1] *The design of a number of units in the apparatus was suggested by Dr. F. S. Brackett, formerly of the National Institute of Health, and the construction was carried out by the personnel of the various shops of the Institute.*

(6) The range of magnification must extend from 20 × up to that reached with oil-immersion lenses. The substage must therefore handle both low- and high-power condensers.

(7) Some type of observation ocular or beam splitter is necessary between microscope and camera to insure correct centering and focusing of the image and to keep it under observation while the apparatus is running. This must be of such type that the limits of the ocular field coincide with those of the motion-picture frame. Sharp focus in the ocular must coincide with that on the film regardless of the objectives or oculars and the projection distance used. The beam splitter must handle satisfactorily the image from objectives without oculars and also that from Homal type oculars. The observation ocular of the beam splitter must be fixed at a height convenient for the seated operator. The unit should therefore be locked on the camera rather than the microscope.

(8) An exact record must be made of the time each photograph is taken. Hence the image of a clock should be inserted in the corner of each frame.

(9) Much of the work with this unit involves runs of 12 hours or longer. In such long runs, it is a common occurrence for the cell or cell groups under observation to migrate out of the field; provision must be made to compensate this migration and to hold the image in the field without any interruption to the film. As any disturbance of the object on the microscope stage would result in interruption or irregularity of the image on the film and as the camera is relatively insensitive to movement, it is more practicable to shift the camera rather than the object. The easiest and most accurate way to change the position of the camera is to use the automatic lateral and transverse shifts of the lathe carriage. This mechanism must be synchronized with the cameras so that at each revolution of the camera shutter the camera moves a predetermined lateral or transverse distance. The camera movement is made possible with the use of the lathe worm screw and its various accessories. The manual controls of the lathe are also used in centering the image in the frame preparatory to starting the camera.

(10) Since the cultures under investigation are almost certainly photosensitive, it is essential that the light be on them as short a time as possible. Except when light is needed for focusing or for forming the image on the film, the cultures should not be illuminated. It was therefore decided to control the exposure by the time the light is on rather than the time the camera shutter is open.

(11) Furthermore, the probable photosensitivity of the cells necessitates the use of relatively rapid photographic film. Hence the exposure must be short or else the image as seen through the observation ocular of the beam splitter is too dim for focusing. In addition to the regular intervals of exposure of the film, some device is needed for turning the lights on for focusing at will during the intervals the camera shutter is closed.

(12) The whole unit must be so constructed that the optical systems are adequately insulated from the floor and from vibration of the motor driving the unit. The microscopes in particular must be carefully insulated since they are most sensitive to vibration. Microscopes and cameras must be rigidly mounted in relation to each other so that no internal vibration or motion will cause displacement or alteration of the image on the film. Normal handling of the apparatus, either for refocusing a lens, recentering the object, or any other such adjustment, must cause no displacement or loss of sharpness of the

image on the film. Hence it was thought best to have the whole double optical system mounted on one concrete mass shaped as a combined base and pillar and to have the motor and gear-reduction mechanism mounted separately; also the air circulator.

(13) All controls, including electric controls, should be within easy reach of the operator regardless of the position of the microscope above the camera.

(14) Means should be provided for regulating the intensity of light reaching the object without altering the diaphragm or condenser settings. This may be done with a Variac type transformer in each light circuit.

(15) Once the correct period is determined for any frequency of exposure, the light must be set to the correct intensity for similar runs, without the necessity of processing trial film strips. Because of the variations of different cultures, a photoelectric exposure meter reading actual light intensities that must reach the film is required.

APPARATUS

Base and Pillar

The base and pillar (figs. 1 and 2) were cast in situ from concrete in a plywood form. The mass was liberally reinforced in all three axes with ½-inch deformed steel rods, and when dry, seasoned, and finished, it was painted. This structure stands with the back of the pillar about 8 inches from one wall of the room. Two 1-inch bolts at the top of the pillar pass through the wall and are insulated from it by 2-inch thick cork pads. The nuts on the bolts are left somewhat loose. Arranged in this way the bolts serve to eliminate any hazard of tilting the unit while in construction, and at the same time they do not transmit vibration from the wall to the pillar. The base rests on pads of Vibracork 2 inches thick, one under each corner: under the pillar is a group of small pads. The space between these pads is filled with softer cork sheets pushed loosely under the concrete mass; the real load, however, is carried by the Vibracork pads, the softer cork merely serving as a reserve cushion to prevent the tipping of the base in case the Vibracork pads should be damaged. Rectangular holes through the base reduce the weight of the unit to floor-load tolerance.

Vertical Lathe-Track Assemblies

The beds from two 10-inch metal cutting lathes 54 inches long were used for the

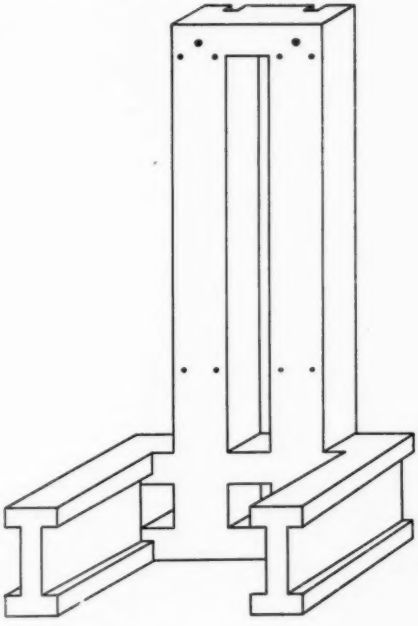

Figure 1.—Base and pillar. Note holes for bolts to anchor pillar to the wall and to attach the vertical lathe beds.

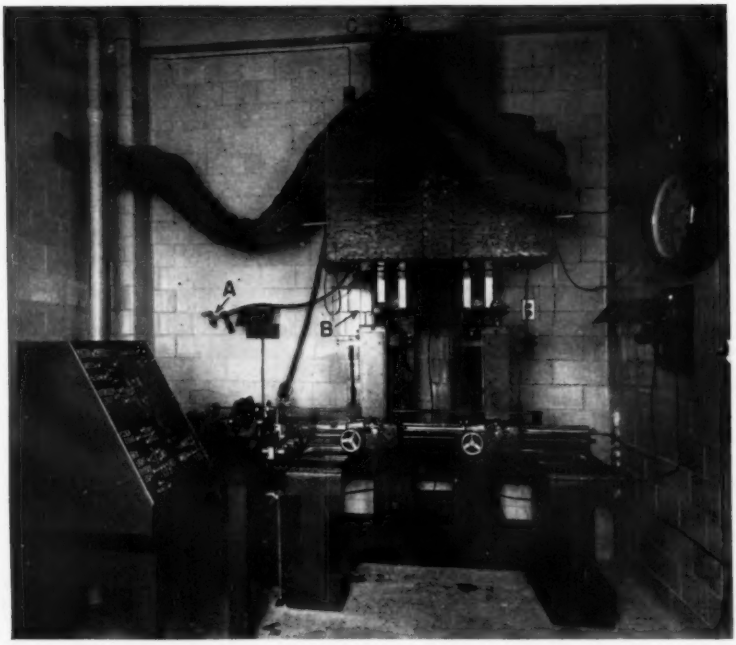

FIGURE 2.—Front view of complete assembly. Insulating doors covering the windows and hand holes of the air bath are closed. *A*, Flexible shaft, with hand wheel for raising and lowering air-bath assembly; *B*, chain actuating carriage lock; *C*, Sheave assembly.

vertical track on which the whole microscope-air bath-light source assembly slides (fig. 2). The two lathe beds were obtained with one carriage each but with no headstock or tailstock assembly, compound slides, or screw cutting worm. One carriage was obtained with complete apron assembly and rack and pinion, the other without this equipment. Both carriages have the usual carriage locks.

The lathe beds are fitted with limit stops so that the carriages cannot run off at either end. In setting up the apparatus, the hand wheel operating the pinion of the rack and pinion on one lathe bed was removed and a brass bevel gear with ½-inch face and a diameter of 4 inches was substituted. To actuate this gear a matching bevel gear 1 inch in diameter is used. This latter gear is mounted in a yoke on the lathe carriage and pinned on the end of a flexible shaft core about 48 inches long. The other end of the shaft core is pinned to the hand wheel, and the lower end of the shaft casing is fitted with a handle (fig. 2, *A*). By turning the hand wheel with one hand while holding the handle of the flexible shaft casing with the other, the pinion working on the lathe rack can be actuated and the carriage moved along the length of the lathe.

The two lathe tracks are mounted vertically on the two vertical members of the pillar. In this position, the flexible shaft that motivates the carriage of the left lathe hangs down alongside the lathe track. The track centers are 17 inches from each other, and the tracks are

shimmed up until roughly parallel and in a vertical position. The carriages are rigidly connected with each other by a cold-rolled steel plate, 1 x 10 x 36 inches, which is laid flat against their surfaces and bolted to them. The two lathe tracks are so adjusted that the carriage and plate assembly will slide easily along them without binding.

The head of the screw actuating each carriage lock is fitted with a double lever arm about 6 inches long, at each end of which a flexible chain is attached (fig. 2, *B*). By a pull on one chain the carriage

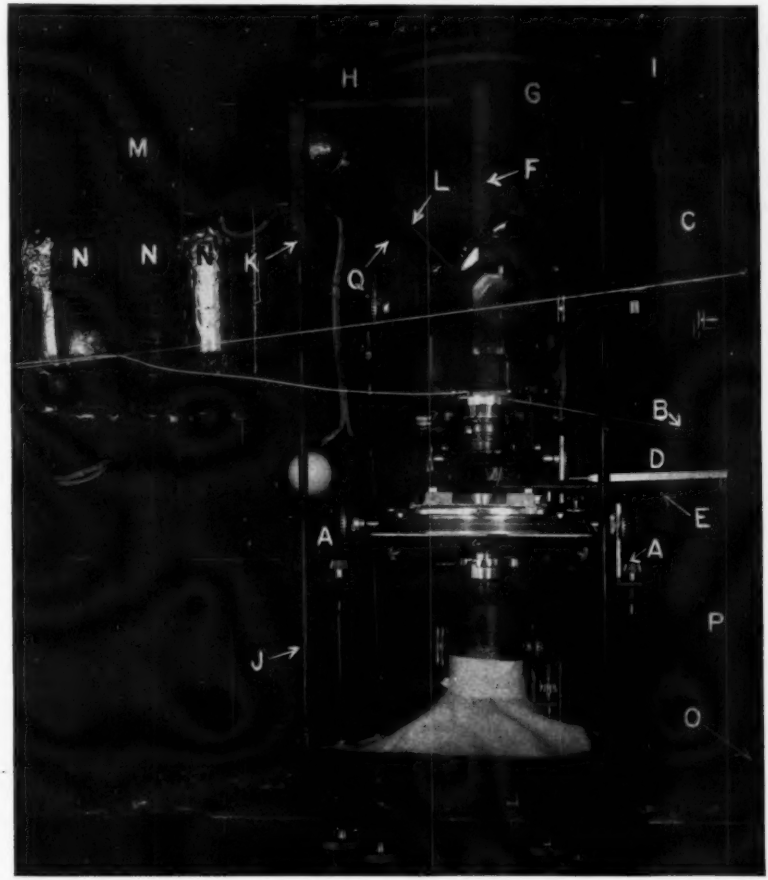

FIGURE 3.—Front view of the right-hand microscope assembly within air bath. Air inlet and outlet ducts and heating unit are also shown. *A*, Bracket supporting gears. When at rest the gears are entirely disengaged; *B*, Linen threads operating iris; *C*, Water cell and filter rack of lamp; *D*, Thermometer; *E*, Thermograph bulb. The thermostat bulb is similarly placed at the other end of the bath; *F*, Substage mirror yoke; *G*, Spur gear carrying mirror yoke; *H*, Metal plate supporting the whole mirror shaft assembly; *I*, Small spur gear controlling mirror rotation; *J*, Shaft regulating the tilt of mirror; *K*, Threaded block supporting mirror-tilt-control block; *L*, Mirror lever arm; *M*, Air inlet tube; *N*, Heater lamps; *O*, Air exit slot; *P*, Air exit duct; *Q*, Clamp holding tiller rope.

can be locked anywhere along the length of the lathe track, while a pull on the other chain releases the lock.

Microscope Bed Plate and Microscopes

The steel plate bolted to the carriages is used as a bed plate for the two microscopes, which are so arranged that the optical axis of each is parallel to the lathe track and 7 inches from the corresponding lateral end of the bed plate. The toes of the microscope bases are about 1 inch from the top edge of the plate, to which the microscopes are securely fastened with bolts through previously drilled holes in the toes and the heel of each microscope base and threading into tapped holes in the plate (fig. 3).

The microscopes are the current Spencer No. 5 model, with rotary mechanical revolving stage and control knobs on both sides of the stage. The special substage rack and pinion have a total vertical range of 6 inches. As in the photomicrographic unit[2] the substage mirrors were lifted off the frames and the stages inverted in our own shop.

The method of extension of the controls of the coarse and fine adjustments, the substage rack and pinion, and the two cross motions of the mechanical stage may be seen from figures 3 and 4. One knurled head was slipped off each of the control shafts of each microscope; the substage pinion and one stage control knob on the left were slipped off the microscope frame on the right; the coarse and fine adjustment heads and one stage control head were slipped off the right side of the microscope. This arrangement eliminates any interference of the extensions with each other and with the camera and permits a distribution of the controls which is convenient for the operator (fig. 4).

[2] Earle, W. R.: *Production of malignancy in vitro. II. Photomicrographic equipment. J. Nat. Cancer Inst., 4: 135-145 (1943).*

For each microscope adjustment shaft, a small L-shaped bracket is used which carries a carefully fitted bearing for the shaft to pass through the vertical portion of the L, while a similar bearing for a 3/16-inch shaft passes vertically through the lower member of the bracket. Once the microscope shaft is slipped through the horizontal bearing, a brass bevel gear 2 inches in diameter with a 3/16-inch face and 64 teeth is fixed on the end of the shaft, where the knurled head was formerly, and holds the bracket on the shaft. This bevel gear is actuated by another with 16 teeth. The second bevel gear is fastened by a set screw on the upper end of a 3/16-inch shaft that is carried out through the lower bearing of the L bracket, through the floor of the hot box, and ends 1 to 3 inches below the floor, the exact length being controlled by the convenience of grouping the shaft ends for the operator. On the lower end of each shaft is an easily removable knurled head 1 inch in diameter (fig. 4, *A*).

The vertical distance from the top of the lower bearing of the L bracket to the horizontal bearing is sufficient so that when at rest the 16-tooth gear does not touch the 64-tooth gear (fig. 3, *A*). To move the 64-tooth gear, the vertical shaft is lifted slightly which engages the teeth of the gears, after which the microscope adjustment can be controlled by turning the vertical shaft. To facilitate the operation and increase the accuracy of this device, two collars are placed on each vertical shaft, one above, the other below, the floor of the air bath. The lower collar (fig. 4, *B*) is set in such a way that when the shaft is raised to enmesh the gears, the collar strikes the lower surface of the air-bath floor and restricts the upward thrust of the shaft, thus spacing the gear teeth the correct distance from each other for easy movement. The upper collar is so set that,

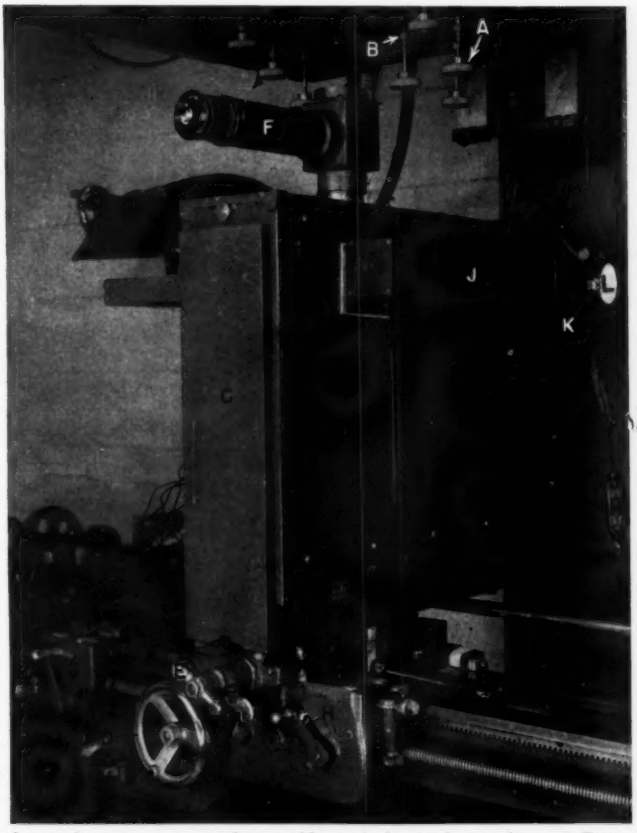

FIGURE 4.—Lathe carriage, camera, watch assembly, and observation ocular. *A*, Extended microscope controls; *B*, Limit collar on control extension shaft; *C*, Saddle supporting camera; *D*, Hand wheel of lathe carriage; *E*, Hand lever controlling transverse movement; *F*, Observation ocular; *G*, Clutch controlling travel of lathe worm; *H*, Clutch controlling lateral motion of lathe carriage; *I*, Clutch controlling transverse motion of lathe carriage; *J*, Watch image mechanism, body tube; *K*, Watch image mechanism, lamp housing; *L*, Watch image mechanism, actual watch.

with the vertical shaft at rest, the weight of the whole shaft assembly rests on the collar which in turn rests on the upper surface of the air-bath floor. All strain on the microscope is therefore eliminated, and when not in use the microscope adjustments are left disengaged.

Where the projection distance from ocular to camera is short, the controls can be operated easily; but where the distance is so long that handling of the controls is tiresome or impossible, the knurled knob is slipped off the lower end of each vertical control shaft, and the shaft lengthened by fastening to its lower end a sleeve carrying an extension of the proper length for easy operation and locking the knurled knob on the lower end of this shaft extension.

Two threads are rigged to each iris lever so that when one or the other is pulled, the iris is opened or closed, as the case may be (fig. 3, *B*).

Air Bath

Figures 2 and 3 show the design of the air bath. The whole bath is supported by clamps bolted on the microscope bed plate. The wall is made from ¾-inch plywood, insulated on the outside with ½-inch celotex; the front has four handholes closed by cloth flaps and has double glass windows inserted wherever possible. The windows and handholes are covered by swinging hinged doors of celotex to prevent further loss of heat, when the apparatus is not in use. The front is hinged at the top to allow periodic cleaning of the microscopes.

The control extension rods pass through accurately centered holes in the floor of the air bath. The ocular and body tube assembly passes through a 3-inch hole (figs. 3 and 4) closed by a chamois sleeve the upper end of which is clamped to the body tube and the lower end sealed to the floor of the bath. This sleeve is so arranged that the whole ocular tube and body tube collar can be slipped out of the body tube from outside the air bath so that the microscope can be used without ocular, or a collar carrying a Homal ocular can be slipped in. There are holes through the end of the bath for the optical systems of the two lamps (fig. 3, *C*), for the linen iris-control threads (fig. 3, *B*), for the insertion of thermometers (fig. 3, *D*), and for thermograph and thermostat bulbs (fig. 3, *E*). There are also holes through the top of the bath for air ducts of the air circulation system.

Substage Mirror Mounting

The substage mirror of each microscope is suspended by diametrically opposed pivots in a yoke (fig. 3, *F*), which is mounted on a spur gear 5 inches in diameter (fig. 3, *G*). The yoke is so placed that the optical center of the mirror coincides with the center of rotation of the gear on its shaft. The spur gear is supported by a short shaft which in turn is suspended from and free to turn in a bearing carried by a metal plate (fig. 3, *H*) attached to the top inner surface of the air bath. The spur gear, which has about 100 teeth, is actuated by a smaller spur gear (fig. 3, *I*) on a shaft similarly suspended from a bearing in the roof plate. This second shaft extends through the air-bath floor, and its end is fitted with a knurled knob which controls its operation. By turning it, the mirror can be rotated on its vertical axis and so adjusted as to illuminate the field evenly.

The tilt of the mirror is regulated by a shaft (fig. 3, *J*) with a control knob below the bottom of the air bath. This shaft passes upward through an arc-shaped slot in the floor of the bath. The finely threaded upper portion of the shaft fits rather tightly into a threaded split block (fig. 3, *K*) supported from the spur gear carrying the mirror. The upper end of the rod presses on a small lever arm (fig. 3, *L*) on the lower edge of the rear of the mirror frame so that, as the rod is threaded in, the mirror is tilted at an increasingly great angle. This lever arm is held against the threaded rod by a small coil spring.

Heater and Air-Circulation System

Some difficulty was experienced in obtaining even heating of the whole active area of the air bath. The system devised might be simplified in minor points, but on the whole it has proved satisfactory.

The air circulator is a small blower (fig. 2) supported by a rubber-insulated bracket on the wall of the room. The bracket also supports the distal ends of two flexible wire-reinforced cloth and rubber hose about 1½ inches in diameter so that the distal ends of the hose clear the inlet and outlet, respectively, of the air circu-

lator by about one-half inch. The hose connections to the circulator inlet and outlet are completed by a single layer of thin rubber dam with a layer of thin cloth over it. Thus transmission of any vibration from the motor to the microscopes is eliminated. The two hose are wrapped with a 2-inch thickness of cotton as heat insulation, and the whole is encased in a light cloth sheath. The other ends of the hose are attached to the air inlet and air outlet, respectively.

The air inlet of the air bath is a 2-inch light metal tube (fig. 3, *M*) which passes down through the roof of the air bath in its midline and near the rear side; its lower end is closed and anchored to the bath floor. About 2 inches from the lower surface of the top of the bath are two rows of half-inch holes to allow passage of air into the bath. Just below them, in a semicircle around the air inlet tube, are four 200-watt tubular projection bulbs wired in parallel series (fig. 3, *N*) which are covered with aluminum foil to eliminate any light. These bulbs are the primary heater unit.

On leaving the bath, air passes through two slots, one at each end of the bath (fig. 3, *O*), into very much flattened ducts (fig. 3, *P*) upward toward the top, and thence into two 1½-inch circular outlet ducts through the roof of the bath. The two outlet ducts (one from each end of the bath) are united and open into a small plywood housing, about 10 x 10 x 10 inches, on the roof of the bath. The housing, which is lined with transite, contains a heater unit similar to that inside the bath. From the housing an air outlet is joined to the flexible hose to the air circulator intake. Both the housing and the ducts on the roof are heavily insulated to reduce loss of heat. They cannot be seen in the figures, although their general shape may be inferred from the contours of the insulation wrapping in figure 2.

To keep an accurate record of the heating of cultures under examination, a thermometer which may be read from outside the air bath is mounted near the stage of each microscope (fig. 3, *D*). A written record of the heat is kept by means of the thermograph on the wall (figs. 2 and 3, *E*). The bulb of the thermograph is at stage level on one microscope, while the bulb of the thermostat is near the stage of the other microscope.

Counterweighting and Support of Air-Bath Assembly

The complete microscope-light-air bath assembly was first carefully weighed; then a thin, rectangular counterweight box was constructed of sheet metal of such dimensions that it could rise and fall freely when suspended in the rear of the pillar. A sheet metal guide was bolted to the pillar so that the counterweight always clears the wall. The counterweight box was filled with sufficient melted lead to counterbalance the whole microscope assembly, and a hook was embedded in the lead near each end of the box. Two flexible steel-wire tiller ropes were attached to the hooks and passed up over front and rear paired sheaves in angle-iron supports on the top of the concrete pillar (fig. 2, *C*). The front sheaves are at such a distance from the front of the pillar that the tiller ropes passing over them extend down toward the air-bath assembly in line with the front-rear center of gravity of the whole air-bath assembly. The ends of the ropes are attached to the assembly by steel clamps attached to the steel bed plate (fig. 3, *Q*).

Projection Distance Scale

In order to reproduce exactly any optical projection distance, the case of a small, spring-controlled metal roll tape, graduated in millimeters, is rigidly attached to

the botton of one of the lathe tracks while the free end of the tape is attached to the lower edge of the air bath. The projection distance is read by means of a pointer mounted on the tape casing.

Camera Bed

The top of each of the two projecting arms of the concrete base is covered with a layer of soft sheet rubber one-eighth inch thick, on top of which is a steel plate 1 x 10 x 15 inches. The lathe bed carrying the camera rests on and is bolted to these steel plates. When so placed, the weight of the bed holds it securely in position, and the rubber sheet protects the microscopes from any slight remaining vibration from the drive shaft.

The lathe bed used to support the cameras is similar to those previously described but is fitted with complete worm-gear assembly, clutch, and change gears used to drive the worm. It has also two carriages, each with complete apron assembly and with the tranverse movement of the tool rest. The graduated hand lever from the front of the lathe (fig. 4) controls this movement. Each carriage is fitted with the usual carriage lock.

Cameras

Model D Universal 35-mm. motion-picture cameras, each capable of taking 400 feet of film (fig. 4), are used. The cameras were first stripped of all unnecessary accessories such as the film dissolve and film reverse, and the shutter of each was locked at 180° aperture. The camera drive, watch-image assembly, observation ocular, and necessary brackets to carry the motor drive were all fitted before being mounted on the lathe carriage.

One camera is mounted in a U-shaped saddle built up from cold-rolled-steel strip, on the transverse slide of each lathe carriage (figs. 2, and 4, *C*). Either camera can be moved along the length of the lathe bed by its lathe carriage hand wheel (fig. 4, *D*), while the front and the rear position can be controlled by the hand lever (fig. 4, *E*) controlling the transverse motion of the lathe carriage.

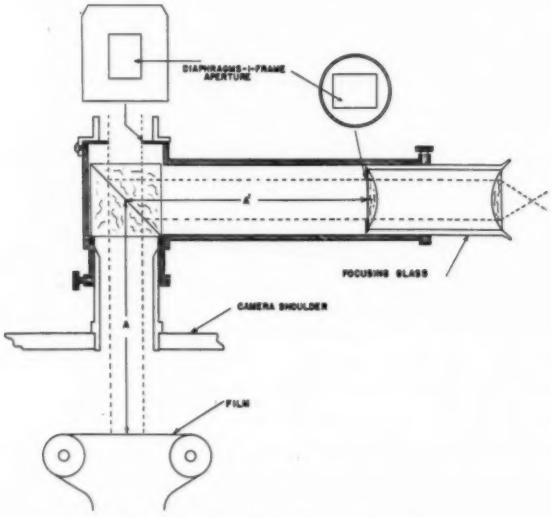

Figure 5.—Observation ocular unit.

Observation Oculars

Figures 4, *F*, and 5 show the general design of the observation oculars. Each ocular consists of a cube-shaped prism, with a 50-mm. face, split and silvered along its hypotenuse so that 10 percent of the light entering the top is reflected to the front, while 90 percent goes through the prism unchanged. The light beam to the front is viewed with a Bausch & Lomb ×3.5 focusing glass adjusted and held in a supporting tube. This glass was selected because of its convenient magnification, and because its lenses are of such diameter that it will give an image that will include all of a 35-mm. frame. The exact limits of the frame were defined by means of a diaphragm, with an aperture the size and shape of one film frame, inserted at the focal plane of the focusing glass.

The ocular unit is mounted on the camera and so adjusted that the field shown in the side eyepiece is identical with that shown in the film. It is adjusted so that the distance from the optical center of the cube prism to the film is the same as that from the center of the prism to the focal plane of the focusing glass (fig. 5, *A* and *A'*). When so adjusted, the image seen through the front ocular coincides with that on the film, both in position and sharpness of focus, regardless of the distance from the microscope ocular to the film. Because of the very large prism held in fixed relation to the camera film, this design of ocular allows the use either of microscope objectives with Homal oculars or of photographic objectives without oculars.

In a few instances internal reflections were noted with the observation ocular when the ocular of the microscope was very close to it. These reflections were eliminated by a limiting aperture diaphragm shaped like a film frame and introduced about 1 cm. above the prism. It blocks out unduly divergent rays from the microscope ocular which would otherwise strike and be reflected from the internal lateral surfaces of the prism.

Some preliminary experiments indicated that one of the chief difficulties that would be experienced in driving the photographic units would be the transmission of vibration from the motor and gear-reduction units to the optical parts of the equipment. After a number of attempts to eliminate the vibration, it was concluded that the most practical way to do so would be in successive steps along the power transmission unit. The power transmission system designed with this in view is shown in figures 6 and 7. The motor is a one-sixth-horsepower, synchronous-wound, heavy-duty type, running at 1,750 r. p. m. under load, and is arranged to drive a Boston type LB20 completely enclosed, reduction-gear unit which reduces the speed to one-twentieth. After a number of commercial couplings were tried, the motor shaft was finally coupled to the closely approximated shaft of the reducer unit by a short length of heavy rubber pressure tubing slipped on the two shafts and clamped at each end to the respective shaft of the motor and reducer (fig. 6, *B*). The motor-and-reducer unit is on a heavy block, which in turn rests on soft rubber cushions at the corner of the block. The block rests on a small table (fig. 6, *C*) completely independent of the rest of the unit and supported from the floor on cork pads. The motor-reducer block is not anchored to the table but can be moved around freely to conform to the position of the gear train in the variable gear reducer.

The slow shaft of the gear-reduction unit is joined by a leather-disk-type flexible coupling to the drive shaft of a flexible gear-reduction unit (fig. 6, *D*, and 7, *A*); the unit is on another table (fig. 6, *E*)

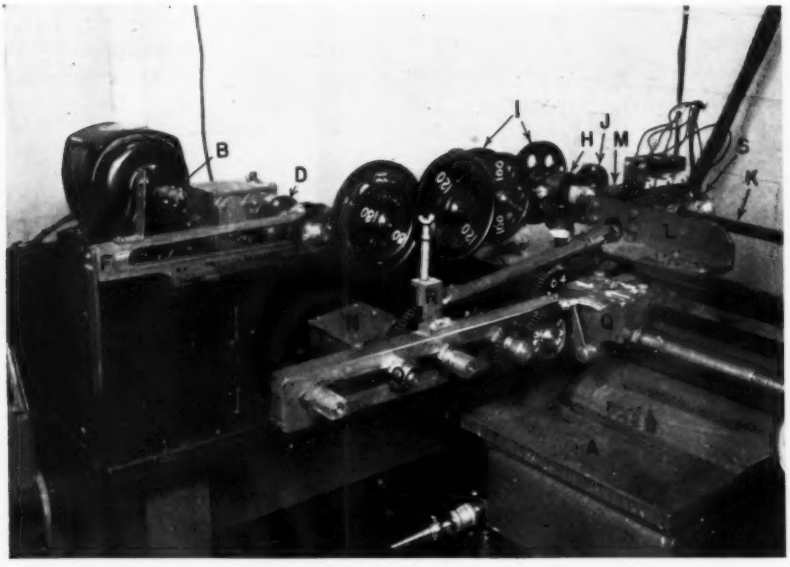

FIGURE 6.—Motor-drive and reduction-gear units, and rotary interrupter switch assembly. *A*, Stee plate; *B*, Rubber tubing coupling; *C*, Table for motor unit; *D* and *J*, Leather flexible couplings; *E*, Table for flexible gear-reduction unit; *F*, Slotted gear support bar; *G*, Support bracket; *H*, Shaft hanger; *I*, Fixed gears to change motor drive from 1,750 to 1,800 r. p. m.; *K*, Keyway shaft running the length of the lathe; *L*, Keyway shaft support and bearing; *M*, Stationary gear box from which automatic shift drive originates; *N*, Gear box driving interrupted gear; *O*, Power-drive shaft carrying interrupted gear (concealed behind support); *P*, Sixty-four-tooth gear actuated by interrupted gear; *Q*, Clutch connecting gear to lathe worm; *R*, Friction-lock device; *S*, Three rotary sector switches actuating micro switches above them.

and is also entirely independent of the photographic unit and the apparatus on the first table. The relative positions of the two tables are shown in figure 6.

The reduction unit consists of a rectangular steel bar (fig. 6, *F*, and 7, *A*) ¾ x 1½ x 30 inches, slotted along its whole length, except for a short distance at each end. Six sliding bearing units (fig. 7, *B*) are locked in the slot. Each of these six units is so designed that it can be locked anywhere along the bar, and each supports a 3-inch length of shafting through its bearing. On each end of these short shafts are compound steel bushings for 20-pitch, ⅜-inch face change gears. By means of the bushings, with necessary spacer collars and lock screws, any combination of change gears can be locked in any one or more of three positions along each of the short shafts. The combination of gears shown in figures 6 and 7, *A*, is one in use at the present time and gives a final exposure rate of one frame to every 30 seconds.

As may be seen from figures 6, *G*, and 7, *A*, the driving end of the gear support rack is fastened to the table by a small bracket, while the power take-off end is supported by a short section of shaft in two shaft hangers bolted to the same table. This shaft passes through a bearing hole near the end of the change-gear support rack.

Since the motor has a rate of about 1,750 r. p. m. and since it is far more simple to

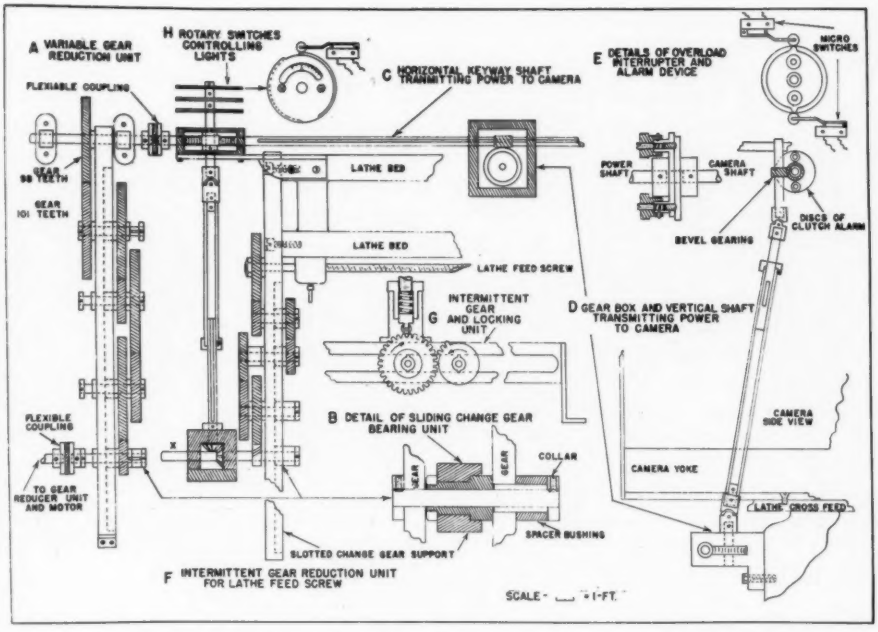

FIGURE 7.—Mechanical drive of entire unit. *A*, Variable gear-reduction unit; *B*, Detail of sliding change gear-bearing unit, *C*, Horizontal keyway shaft for transmitting power to cameras; *D*, Gear box and vertical shaft for transmitting power from keyway shaft to camera; *E*, Details of overload interruption and alarm device; *F*, Intermittent gear-reduction unit driving lathe feed screw; *G*, Intermittent gear and gear-locking device; *H*, Rotary switches controlling lights.

calculate gear ratio necessary when a rate of 1,800 r. p. m. is used, the two gears (figs. 6, *I*, and 7, *A*) nearest the power take-off end of the gear support bar were selected with 101 and 98 teeth, respectively. With the use of these gears, the ratio is stepped up to where the motor may be considered as running almost exactly 1,800 r. p. m., which greatly simplifies calculations for gear combinations. By locking different combinations of gears in this gear support bar, a wide range of speeds is obtained. In use, no trouble is experienced in working from one revolution per second to one per minute, while an even wider range of slow drive speeds is obtained by substituting a lower ratio fixed gear-reduction unit, for instance, the LB 100, for the LB 20 unit used.

The power take-off shaft from the flexible-gear-reduction unit is joined by means of a leather flexible coupling to a ⅝-inch shaft (figs. 6, *K*, and 7, *C*) running the length of the lathe bed. The shaft is supported at each end by a steel bearing attached to the rear of the lathe bed (figs. 6, *L*, and 7, *C*); the cameras are driven from this shaft, which has a ⅛-inch keyway running its entire free length.

The drive connecting the keyway shaft with each camera drive, shown in figures 2, and 7, *D*, allows the cameras to move laterally and transversely without loss of synchronization with each other or with

the adjustments of the rotary light switches and the automatic camera shift. To permit such movement of the cameras, a small gear box (fig. 7, *D*), through which the keyway shaft passes, is mounted at the rear of each camera. Within each gear box are mounted a worm and a worm gear; the worm is on the keyway shaft along which it slides freely, but it is kept from rotating around the shaft by a key fitting the keyway of the shaft and traveling with the worm. The worm drives the worm gear, which in turn is pinned to the lower end of the camera drive shaft that goes up through the top of the gear box. Just outside the gear box, the shaft ends in a Curtis universal joint, from the upper end of which runs a telescoping shaft. The upper end of the telescoping shaft carries another Curtis universal joint which links to a short section of vertical shaft supported on a bracket from the camera. Figure 2 shows this bracket. The vertical shaft carries a steel spiral gear engaging another spiral gear on the extension of the camera drive shaft, and the latter in turn drives the camera. The telescoping shaft in this drive allows the camera to be moved by the transverse movement of the lathe carriage without interfering with the power drive, while the sliding of the gear box along the keyway shaft permits the lateral shift of the camera units.

Shaft Overload Release and Alarm

A shaft overload safety release (fig. 7, *E*) is inserted in the shafting just before it enters the camera. This release consists of a 2½-inch brass disk on the end of the shaft from the motor drive just outside the camera; on the end of the shaft leading into the camera is a similar disk. Through the disk on the drive end are two small spring plungers, with hemispherical ends which fit into shallow hemispherical depressions in the side of the other disk. As long as the load that the camera exerts on the drive is not excessive, the plungers keep the two disks locked together so the camera drives uninterruptedly. However, should the film jam, the load on the plungers becomes excessive and they slip out of their depressions; the disks are then no longer locked together, and the drive disk rotates in relation to the camera disk. Since the two pins holding them are at different distances from the centers, the pins cannot re-engage the depressions until the disks have traveled through 360° in relation to each other. If the jam continues, they again slip.

Each disk is milled so that approximately 170° of the circumference is one-sixteenth inch lower than the remaining 190°. While the pins rest in their depressions, the combined circumference of the joined disks is made up of the 190° portion of each. When the pins disengage their depressions because of jamming the camera and the disks slip in relation to each other, their combined circumferences are no longer at the 190° level, but at least some part is at the 170° level. This change in level serves to release one of the two small micro switches bearing on the combined circumferences, thus closing an electric circuit and sounding an alarm bell. These micro switches are mounted on the side of the cameras, and the two switches on any one camera unit are wired in parallel so that either controls the alarm.

Automatic Shift Drive

Near the power drive end of the keyway shaft supported along the rear of the lathe bed is inserted a stationary gear box (figs. 6, *M*, and 7, *F*) containing a worm that is rigidly pinned on the keyway shaft, and a worm gear. This mechanism drives a telescopic shaft extending outward toward the front of the apparatus. The front end of the shaft enters another small

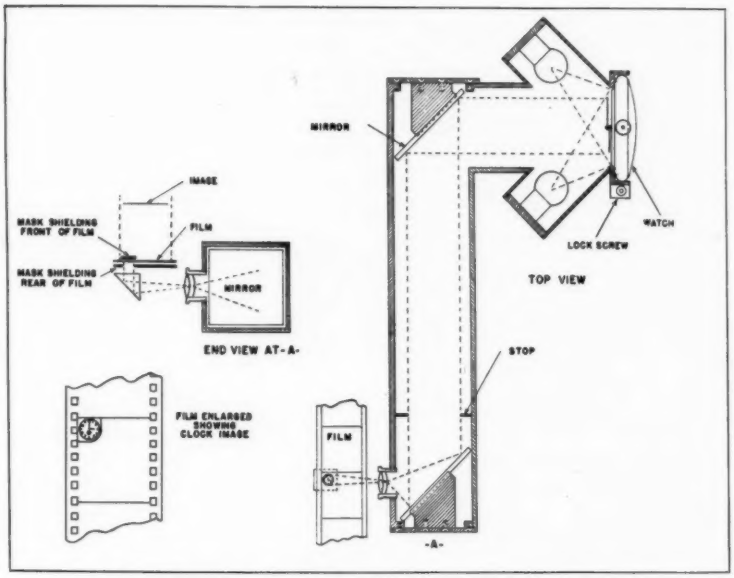

FIGURE 8.—Watch-image mechanism, front and top views.

gear box, where a miter gear is pinned on the end of the shaft, and this in turn drives another miter gear on the end of a shaft (fig. 6, *O*) that extends out parallel with the keyway shaft driving the cameras. Tihs shaft is the power-drive shaft that actuates another variable gear-reduction unit similar to but not so large as that used on the whole camera drive. Attached to the far end of this shaft is a 20-tooth change gear from which all but 3 teeth are removed (fig. 7, *G*). This interrupted gear actuates a 64-tooth gear (fig. 6, *P*), which in turn works through other gears in the chain and through a clutch (figs. 4, *G*, 6, *Q*, and 7, *F*) and finally drives the worm screw on the lathe. These gears are so selected that each revolution of the interrupted gear results in a lateral travel of the lathe carriage, of $\frac{1}{1000}$ inch, whereas the interrupted gear is synchronized with the rest of the drive mechanism in such a way that this movement takes place only while the shutter of the camera is closed. All these gears are from the original change gears furnished with the lathe.

When this mechanism was first used, some little trouble was experienced from backlash, which caused the gear actuated by the interrupted gear to slip a little during the interrupted interval and so jam the mechanism. Backlash was eliminated by fitting the gear with a friction-lock device (figs. 6, *R*, and 7, *G*), which holds it in position until it is re-engaged at the next revolution of the interrupted gear.

Once the mechanism is adjusted, the worm can be moved as desired, with the lathe clutch controlling the worm drive; if the clutch on either lathe carriage is then engaged, that carriage can be made to travel automatically in either direction laterally (fig. 4, *H*), or by engaging the transverse clutch (fig. 4, *I*) of the lathe carriage the camera can be moved toward either the front or the rear of the lathe. With the lathe design used, the transverse

and lateral movements can be effected simultaneously. With the use of this automatic shift for one or more short intervals during the course of a reel, a clump of cells once centered can be followed for any interval desired, while the movements of the camera are smooth, without vibration or disturbance of focus, and do not interfere with the continuity of the film.

Watch-Image Mechanism

Since the positions of the two cameras are not fixed in relation to each other, a watch unit is mounted on each camera so that the image of the watch may be inserted on each frame of the film. The general positions of these units are shown in figure 4, *J*, *K*, and *L*, and the design in figure 8. The small prism reflecting the image onto the film is mounted directly on the film gate. The lens used in forming the image is the rear lens of a 4-mm. microscope objective and is threaded directly into a hole tapped in the door of the camera. The light source is miniature electric globes; the timepieces are cheap watches with crystals removed, each watch being held in an easily released clamp so that it can be taken out for setting and winding. The two watch faces are marked A and B, respectively, to show on the film with which camera the film was taken. When a film is ready to be started, a small gummed number label (Dennison's No. 20 C) showing the number of the film being run is attached to the face of each watch so as not to interfere with the numbers on the face or with the hands. The watches are synchronized with each other and with the electric clock on the wall (fig. 2) and are locked in their respective camera units.

To prevent the formation of a double image, one from the watch and the other from the microscope ocular, in the area where the image of the watch is projected on the film, a mask is inserted in the regular mask orifice of the camera. The mask is open, except for a small area including only the image of the watch. This area of the watch is blocked so that no light from the microscope ocular can here reach the film. Another mask, blocked to exclude light from all sources except this watch area, is inserted in the back of the film gate to protect the rear side of the film.

Mechanism for Controlling Duration of Exposures

The onset and duration of exposure for both microscope lamps and both sets of clock lights are controlled by two adjustable rotary sector switches (figs. 6, *S*, and 7, *H*) on a short rear extension of the automatic shift drive. The switches are synchronized to turn the microscope lights on during the time the camera shutters are open and consequently during the interval the film is motionless.

Each sector switch consists of two metal disks about one-sixteenth inch thick and about 4 inches in diameter. The circumference of each disk is milled so that 180° is one-sixteenth inch lower than the rest. One of the disks is rigidly locked on its shaft; while the second is locked, not on the shaft, but to the first disk by a bolt in the latter which passes through a slot in the former and has a nut on it. The two disks can thus be locked at any angle of rotation around their common axis. By adjusting the overlap of the high and low parts of their circumferences, the low part of their combined circumferences can be adjusted to any arc from 0° to 180°, the exact arc being determined by a graduated scale and vernier, respectively, on the disks. Above each pair of disks is a small micro switch [3] with a lever arm carrying a roller bearing on its end that rests on the cir-

[3] *Made by the Micro Switch Corp., Freeport, Ill.*

cumference of the sector disks, so that as the latter turn, when the roller bearing on the lever arm enters a low place in the sector disks it actuates the switch and turns on the light. The light continues to burn as long as the roller is in the low area; but when it strikes the high area in the sector, the switch opens. The sectors are synchronized on the shaft so that the disk locked on the shaft determines when the roller enters a low part of the circumferences and is synchronized with the opening of the camera shutter, while the second disk determines when the roller will leave the low part and thus how long the light is on.

One sector-disk switch controls both clock lights, the second controls both microscope lights while the shutter is open. A third sector switch on the same shaft is connected with the microscope light circuit so that during each complete closure of the camera shutter the microscope light is thrown on and held until the shutter is ready to open. This interval of illumination permits accurate checking of the focus and position of the object under the microscopes without interrupting the running of the film. When not in use, the third sector switch can be cut out of the circuit by means of a small hand switch on the electric control panel (fig. 9). Using micro switches for opening and closing these light circuits has proved a simple and satisfactory solution for a difficult problem in the construction of the apparatus. During more than a year of use, there has been no instance of failure of a switch or of uneven lighting because of defective switch function in the sequence of any film.

Electric Control Panel

The electric control panel is shown in figure 2 and diagrammatically in figure 9. In addition to a master switch which controls all circuits, separate switches are provided for the motor and the motor-drive

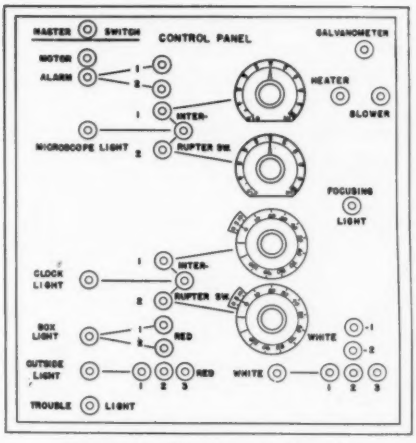

FIGURE 9.—Electric control panel.

overload alarm, and subordinate switches for each camera. The microscope-light-switch system includes a general switch and one to cut the rotary-sector-disk switch in or out of the circuits. Apart from all other switches is the switch to throw the focusing light interrupter in or out of circuit. The switch system on the clocks is essentially similar to that for the microscope lights, but there is no focusing-light switch. Since the lights are of low wattage, rotary variable resistances are used instead of variable transformers for controlling light intensity. Additional switches on the lower end of the panel control illumination within the air bath and in the outside room; both deep yellow safelights and white lights are provided. Switches for the latter lights are segregated. The switches at the upper right hand corner of the panel control the photoelectric galvanometer and the air-bath heater and blower. The circuits of these switches are not controlled through the master switch.

Photoelectric Unit

Because of the variable optical conditions prevailing in different cultures, the photoelectric system described for our still cam-

548963—43——3

era [4] is used for determining the light intensity and duration of exposure with this equipment. The photoelectric cell is merely laid on top of the camera alongside the observation ocular, and the camera racked laterally until the cell is centered under the microscope ocular. Then, by means of the variable resistance in circuit with the microscope lamp, the light reaching the film may be set at any desired predetermined intensity. This operation must, of course, be carried out with the room dark. The photoelectric cell is connected with the same galvanometer system as that used for our still camera.

[4] *See footnote 2.*

Production of Malignancy in Vitro.

IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells

By WILTON R. EARLE, *senior cytologist*, with the technical assistance of EDWARD L. SCHILLING, THOMAS H. STARK, NANCY P. STRAUS, MARY F. BROWN, and EMMA SHELTON,
National Cancer Institute, National Institute of Health, United States Public Health Service

INTRODUCTION

Earle and Voegtlin (*1*) showed that 20-methylcholanthrene added to culture fluid as a fine suspension in concentrations ranging from 1,000γ to 0.2γ per cubic centimeter, has a definitely injurious effect on cultures of subcutaneous rat and mouse fibroblasts grown in chicken fibrin clot with a supernatant-fluid culture medium of horse serum and chick-embryo extract diluted with saline solution. The carcinogen caused partial or complete necrosis, particularly at higher concentrations, and always caused a retardation in the rate of growth. The study was carried to 54 days after the first addition of the carcinogen.

Using the same culture medium and fibroblasts from the strain C3H mouse, Earle and Voegtlin (*2*) continued the study with a concentration of 10γ of carcinogen per cubic centimeter of culture fluid for periods up to 250 days and found less degeneration than was shown at the higher concentrations (*1*), but there was the same severe initial retardation of the increase in size of the clump. Cultures that had received the agent as long as 114 days showed a change in the architecture of the clump to a distinctly more epitheliallike type, which seemed to result from marked changes in the cells, at least some of which was in the cell surfaces. The cells became laterally coherent; this change was gradual, progressing with prolonged exposure to the carcinogen and affecting the culture as a whole rather than a few isolated cells.

Unfortunately, the type cultures did not allow an accurate analysis of the cell changes in the early stages. Changes after 265 days in methylcholanthrene were also not investigated, owing to loss of the series from a bacterial contamination. This loss cut short studies on the behavior of the carcinogen-treated cells on reinjection. Only 12 C3H mice were injected with 1 culture each of cells that had been subjected to the carcinogen about 114 days and then carried 83 days longer without addition of the carcinogen. The results of these injections were negative.

Just before the series was lost, it was noted that the control cultures were showing a trace of the same morphologic change as that in cultures of the treated cells. This was ascribed to trace contamination with methylcholanthrene, which could possibly have occurred with the technique used.

The studies were later extended, with four general aims:

(1) To confirm the previously observed cell changes with a more accurate control of the concentration of methylcholanthrene.

(2) To follow with greater accuracy the progression of changes in the cells, both in the carcinogen and after removal from it, with particular attention to the sequence of changes during the first 60 days after exposure to the carcinogen

(this interval corresponding approximately to the latent interval for production of tumors by injections of the carcinogen in vivo) and the cell changes after exposure for more than 260 days.

(3) To attempt to demonstrate conclusively a change from the normal to the malignant cell by the action of the carcinogen in vitro. This of necessity involved the production of tumors by injection of cells treated with the carcinogen in vitro.

(4) To correlate the relative tumor-producing ability of the cells with morphologic changes observed in them.

Although these studies are as yet incomplete, the present paper reports the technical methods used and the data accumulated on a primary strain of mouse fibroblasts, cultures of which were carried in methylcholanthrene for various intervals up to 406 days. While the general sequence of morphologic changes as observed in the living culture is given, this paper does not include any work with stained preparations. A description of the production of the tumors obtained by inoculation is given in the next paper (*3*), while the pathology of the tumors will be presented in the paper following (*3a*). Other studies in the series will appear in later issues of this Journal. These will cover (1) observations on the mitochondria and Golgi apparatus, and (2) metabolism of the tumors produced. Data from other stained preparations, from motion picture reels, and analysis of the growth rates of the tumors are as yet incomplete and will be presented at a later date. The equipment and method used in cleaning the glassware and the photomicrographic and the microcinematographic equipment designed for use in these experiments are described in the three preceding papers (*4, 5, 6*).

MATERIALS AND METHODS

In addition to the acid cleaning equipment and method already described (*4*), this investigation has necessitated the construction of considerable special apparatus and the elaboration of specialized procedures. Sufficient details are given to make clear the course of the experiment, as well as the various precautions taken.

Cleaning of Equipment

All glassware on hand was first cleaned by using the acid-cleaning method (*4*). Then the glassware, used with normal-tissue-culture solutions only and not contaminated with heavy metallic salts or with carcinogenic substances, was washed with soap and water as usual, rinsed with distilled water, and drained.

With the single exception of the cultures that were fixed in situ in Carrel flasks with Orth's formalin-dichromate solution, the glassware used with tissue cultures was never employed with any solution for fixation or staining. After fixation of cultures in the Carrel flasks, the flasks were given a preliminary cleaning in a mixture of equal parts of concentrated nitric and sulfuric acids at 80° C. for 24 hours and then, after being drained at a sink reserved for waste acids, were rerun through the regular acid cleaning bath. In this way, there was no contamination of the large acid cleaning baths with chromium, and yet the carcinogen was destroyed. In flasks thus cleaned, cultures have grown satisfactorily and have shown no sign of the irregular and poor growth so common in those grown in glassware cleaned with dichromate-sulfuric acid.

All waste carcinogenic solutions were destroyed by sulfuric acid in the acid bath if exceedingly concentrated; but if the concentration was not more than 1γ per cubic centimeter, the solutions were flushed down a small covered sink used for that purpose only.

After being cleaned, all glassware and other materials were wrapped in paper and autoclaved at 20 to 22 pounds steam pressure for 1½ hours, or dry sterilized at about 170° to 175° C. for 3 to 4 hours, after the desired temperature was reached.

Rubber stoppers and rubber equipment that might come in contact with the culture fluid were boiled repeatedly with an approximately 5-percent solution of sodium hydroxide, then washed

free from alkali, and rinsed repeatedly with distilled water. An attempt was made to have as little rubber as possible come in contact with culture solutions. All rubber equipment was sterilized by autoclaving.

Until about June 8, 1942, all rubber items, including stoppers, tubing, etc., employed with solutions containing carcinogen or with carcinogen-treated cultures, were used once and then destroyed. Since then because of the critical shortage of rubber, stoppers used with cultures removed from the carcinogen for at least 60 days, are cleaned with sodium hydroxide solution as described and used over for such cultures. They are never used with untreated cultures.

The dissecting knives and needles used in transferring carcinogen-treated cultures to fresh flasks were cleaned by soaking them in five to six consecutive changes of acetone and benzene. They were then sterilized with dry heat at 170° C.

Distilled Water

All distilled water used in rinsing glassware was from the general distilled water system of the laboratory. All solutions used with the cultures were prepared from this distilled water, which had been carried through two additional distillations in a double all-glass still and collected and stored in 5-gallon pyrex bottles. The rubber stoppers used with the bottles were cleaned by repeated boiling in sodium hydroxide solution. Care was taken that the distilled water did not touch the rubber.

Isotonic Saline Solution

The saline solution used for washing cultures and for diluting culture media consisted of the following ingredients:

	Grams
Sodium chloride	6. 80
Potassium chloride	. 40
Calcium chloride	. 20
Magnesium sulfate	. 10
Sodium dihydrogen phosphate	. 125
Sodium bicarbonate	2. 20
Dextrose	1. 00
Glass-distilled water to 1,000 cc.	

All concentrations were calculated in terms of anhydrous salts, which were of reagent quality and were purchased in relatively large lots so that there was no change in lot number for most of the salts used. With the exception of the calcium chloride, the salts were dissolved, in the order given, in about 600 cc. of water for each liter of solution. The calcium chloride was kept in the form of an analyzed stock solution containing 0.1 gm. anhydrous salt per cubic centimeter. The correct amount of this solution was added to 100 to 200 cc. of water for each liter of final solution, and this in turn poured into the rest of the solution with agitation. The pH of the solution was set at 7.4; if necessary, a little carbon dioxide was run through it from a cylinder of compressed gas. The solution was then filtered. Inasmuch as no such physiologic saline relying on bicarbonate for its buffer action can be filtered under vacuum without a severe shift in pH to the alkaline side, all filtrations were carried out by using air pressure at 3 to 5 pounds. The filter candles employed were never used for any other purpose. During the early part of the experiment the solution was made up and filtered in lots of 4 l. through a 1-by-5-inch Mandler candle of normal porosity designed to handle 7 to 9 pounds of air pressure; later the solution was made up in lots of 32 l. and was simultaneously filtered through two 2-by-10-inch Mandler candles to give two final lots of 16 l. each. The filtering equipment is shown in figure 1, *A* and *B*.

In autoclaving such large units as the 32-l. saline apparatus, the stopcock permitting access of air to the apparatus was wrapped but left open, while in each of the two large containers about 100 cc. of glass-distilled water was placed, thus insuring the formation of enough steam within each container to act as a sterilizing agent. These units were autoclaved at 20 pounds for 2 hours and then allowed to dry out. After filtration, the saline solution was run into flasks which were sealed with rubber stoppers and stored at 3° C.

During the whole interval of preparation and storage the pH of the saline solution would rise about 0.1 unit. Since the solution was desired at pH 7.5, it was originally set at about pH 7.4. The pH of the other solutions was similarly controlled so that the pH of the final culture media was about 7.5.

Horse Serum

While no particular selection was made as to the age and sex of the horses from which the serum was obtained, records show that all the horses were adult geldings ranging up to 33 years of age. Most of the serum was from horses estimated to be about 16 years old. All serums were clear, and no trouble was ever experienced from hemolysis.

The jugular vein was punctured with a 4-mm. (outside diameter) Graefe trocar, with stopcock, connected with a rubber tube, and the blood collected in 3-1, lots in sterile glass cylinders about

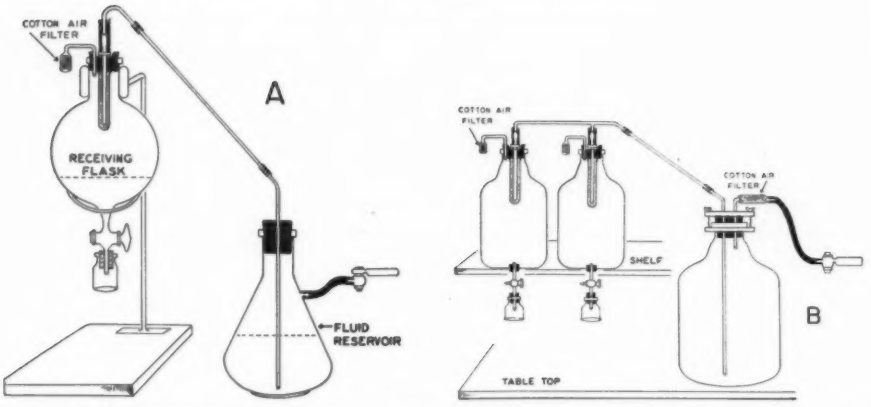

FIGURE 1.—*A*, Equipment used to filter saline solution and horse serum in lots of 4 l. or less; *B*, Equipment used to filter saline solution in lots of 32 l. The small bottles are of 5-gallon capacity, the larger one is of 12-gallon capacity. All are made of pyrex.

100 mm. in diameter and 450 mm. high, which were kept cool in crushed ice to reduce changes in the blood. Usually 9 l. was obtained from one horse. When horses were bled on Saturday and allowed to rest over the week end, the bleeding did not interfere with their regular work. The blood was allowed to clot, and the clots were loosened from the cylinders which were held at 3° C. overnight. During this handling sterility was not maintained although an attempt was made to keep the blood reasonably clean. Next day, the serum was drawn off with a 200-cc. bulb pipette. The clots were minced with scissors, the fragments were centrifuged, and the expressed serum was removed. The total lot of serum was recentrifuged to eliminate any remaining red cells which would be disrupted by freezing, then pooled, and stored in 1,000-cc., flat-bottomed, boiling flasks sealed with rubber stoppers. The flasks were filled not more than half full to prevent their breaking on freezing, then stored at —10°, and held frozen until needed. When needed, the serum was thawed out, recentrifuged to remove any fine precipitate that might (very rapidly) clog the filter, and filtered under pressure through a 1-by-5-inch Mandler candle. The smaller equipment and the method for filtering saline solution were used. The filtered serum was stored at 3° in small flasks sealed with rubber stoppers. Since the filter candles were used repeatedly, they were carefully washed with a dilute sodium chloride-sodium bicarbonate solution, then with glass-distilled water. Filters used for horse serum were reserved for that solution only. The serum when used was usually less than 60 days old. In other work, serums up to 9 months old have been used without any observed deleterious action.

PLASMA

Plasma was obtained from hens weighing 5 to 8 pounds and not more than 1½ years old. It was necessary to watch carefully the age of the hens, as the use of plasma from older hens has been found to result in poor growth of the cultures. A simple glass cannula sterilized in heavy mineral oil at about 140° C. was inserted in the carotid, and the blood collected in a narrow-mouthed, heavy-walled, iced, paraffined, 100-cc. centrifuge flask, which was stoppered with cotton. The surplus melted wax from the tube had been drained into the cotton. Great care was taken not to overheat the mineral oil and wax. The blood was centrifuged in ice for 5 minutes at 3,000 r. p. m.; the plasma was drawn off with a 5-cc. pipette and stored at 3° in a paraffined, cotton-stoppered, 50-cc. centrifuge flask. This plasma was used within 30 days. No anticoagulant was used. While some tubes were lost because of spontaneous clotting, the loss was never so severe as to necessitate the use of heparin.

EMBRYO EXTRACT

Embryo extract was prepared from 9-day chick embryos. The whole unwashed embryo was minced by running it through a 30-cc. Luer syringe in the lower end of the barrel of which was a 28-mesh monel-metal wire gauze. The

minced tissue was received in a 100-cc. narrow-throated centrifuge flask wrapped integrally with the barrel of the syringe. Approximately one volume of saline was added and the whole agitated briefly by sucking it in and out of a 25-cc. wide-mouthed bulb pipette. The flask was rubber stoppered and centrifuged at 3,000 r. p. m. for 20 minutes. The supernatant fluid was pipetted into 50-cc. centrifuge flasks, rubber stoppered, and frozen at once with carbon dioxide snow to kill any living cells. The solution was kept frozen until needed, at which time it was thawed out and recentrifuged at 3,000 r. p. m. for 20 minutes to clarify it directly before use.

Embryo extract was prepared two, or more usually three times a week. An attempt was made to use it when less than 5 days old, but in a few instances as a result of some accident older extract was used for a single change of fluid on the cultures.

Of the various culture media used, the embryo extract was probably subject to the greatest variation, both from lot to lot of eggs and possibly from season to season. In some earlier work in which refractometer readings were made on different lots of embryo extract, a wide difference was found in lots prepared on different days. Because of these variations, particular care was taken to use the same lot of embryo extract for each experimental culture and its control.

Preparation of Methylcholanthrene

The 20-methylcholanthrene used was all of the same lot, which was different from the lots used in earlier work (*1*, *2*). It was purified by Dr. J. L. Hartwell, of this Institute, and had a melting point of 179.3°–180.0° C., corrected.

Whereas in the previous work methylcholanthrene had been used in the form of a fine suspension, in this experiment it was dissolved in the serum. A lot of 2 l. of serum was placed in a glass-stoppered 4-l. pyrex bottle; about 40 mg. of the crystalline methylcholanthrene was weighed out into a small agate mortar, one or two drops of serum were added, and the mixture was rubbed to an extremely fine, creamy paste; more serum was added and the suspension transferred to the serum in the bottle quantitatively. The serum was held at 3° C. for 24 hours and shaken occasionally, and then shaken in a mechanical shaker for 24 hours. It was next run through filter paper to get out any particles that might clog the filter, and then filtered, through a Mandler candle into flasks that were sealed with rubber stoppers. The Mandler candle was used once, then destroyed.

This method of solution of the carcinogen was suggested by Dr. Egon Lorenz, of this Institute. Dr. Lorenz also analyzed spectroscopically the first lot of solution. The determination showed a concentration of between 2.0γ and 2.5γ of methylcholanthrene per cubic centimeter. Later lots were not analyzed but were prepared with the same technique and were considered to have approximately the same concentration.

Although an attempt was always made to have the relative size of the control and carcinogen-containing lots such that they would last for an equal period, it was rare that the two lots ran out at exactly the same time; usually one was exhausted a few days ahead of the other. Since no differences have been detected in the action of different lots of serum prepared by the method used, no hesitancy was felt in using up the last few flasks of any lot. To avoid any hazard of accidentally using a carcinogen-containing solution in normal cultures, the carcinogen-containing solution was always segregated. As a further precaution, the container of the normal solution carried a blue or black label, that of the carcinogen-containing solution a red label.

Duplication of Cultures

To prevent the loss of a whole strain of cultures through bacterial contamination of a stock solution, duplicate independent lots of solution were used, and all culture series were so divided that contamination of any single solution would result in the loss of only half of any one series or strain of cultures. No such contamination of a stock solution has occurred.

Carrel Flasks

All cultures were prepared and carried in modified Carrel type D flasks made of pyrex and having an outside diameter of 33 to 34 mm., a height through the flat area of approximately 10 mm., and floor and roof thickness of about 0.4 to 0.6 mm. This thickness gave good optical definition with a 16-mm. objective, yet the flask was not so fragile as to be hazardous when used with carcinogenic solutions. The throat length was approximately 33 mm., measured from the juncture of the throat with the top of the flask; the throat had a wall thickness of 1.0 to 1.5 mm. and an inside diameter of 10.0 to 10.5 mm. These changes in the throat were necessary to permit the sealing of the flasks with standard size 00 rubber stoppers.[1]

[1] *Flasks of this type may be obtained from E. Machlett & Son, 220 East 23d St., New York, and from Hopf Glass Apparatus Co., 192 3d Ave., New York.*

Incubation of Cultures

All cultures were maintained at a temperature of approximately 38.2° to 38.8° C. in an incubator having an ample water jacket. The heaters in the incubator warmed the water jacket rather than the internal air space, while the two thermoregulators, which were wired in series, were immersed in the water bath. In this way sudden overheating of the incubator for a short period after the doors had been opened and closed again was eliminated. One thermoregulator was adjusted for a temperature 0.3° higher than the other so that it did not function at all but merely served as a reserve regulator. An alarm bell was wired into the circuit of this reserve regulator. The air in the incubator was subjected to forced circulation to insure even heating. In the incubator, the culture flasks were arranged in racks of 10 (fig. 2, *A*) and placed on shelves hinged at the left end, while a motor mechanism mounted on the roof of the incubator alternately raised and lowered the right end of the shelves through a cycle of about $\pm 3\frac{1}{2}°$ from the horizontal at a rate of six cycles per hour. This caused the culture fluid in the flasks to be washed over the surface of the clots and helped to eliminate local changes in the culture medium within the flasks. The racks held the flasks level. If the racks were ever soiled with carcinogen, they were destroyed.

A temperature of about 38.5° C. was maintained during the visual examination of the cultures by having the microscopes surrounded with a thermoregulated air bath sufficiently large to take several racks of cultures. The photographic equipment was similarly held to constant temperature. With these precautions, the only serious intervals of lowering the temperature were when the cultures were reduced to room temperature during the three weekly changes of culture fluid. These intervals of cooling averaged about 4 hours each time and were constant for the whole series. It has as yet been technically impracticable to eliminate them.

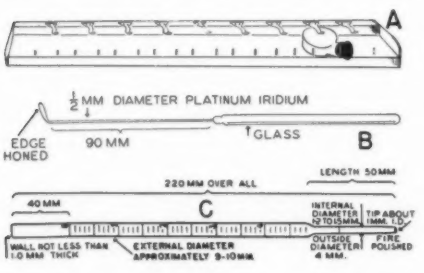

FIGURE 2.—*A*, Flask racks, made of wood with metal ends, and springs of sheet phosphor bronze 0.25 mm. thick. The pins are brass escutcheon pins; *B*, Platinum-iridium spatula used to transfer cultures. The curved portion of the spatula is flattened by hammering and then honed to a sharp edge; *C*, Pipette for handling small lots of solution like plasma.

Light

To eliminate complications that might result from the action of short wavelengths of light on cells photosensitized by methylcholanthrene, all cultures were handled, examined, and photographed by orange light. All light sources of general illumination in the rooms were shielded with five layers of No. 300 Tango shade of cellophane. The microscope lights were 6-volt, 108-watt, ribbon-filament, incandescent bulbs shielded with water cells and deep orange filters (Corning No. 351), cutting off at about 500 mμ. For photomicrography where the light was concentrated on the culture by a condenser, the light was further shielded with a Corning No. 397 heat-absorbing glass filter. While it cannot be said that no white light ever reached the cultures, the total amount reaching them during the whole course of the experiment was extremely low.

Preparation of the Original Explant

The original explant tissue was dissected out, cut up rapidly into strips about 2.2 x 6.0 mm., and placed in saline in depression spot plates. The cultures were then planted as described in the following section.

Transfer of Cultures

Cultures were transferred to fresh flasks about every 27 to 35 days. The series of flasks containing the cultures were arranged on the table in order of transfer; each flask was flamed very hot around the throat, the stopper removed, a sterile cork inserted lightly, and the flask set aside to allow the throat to cool. By the time the last of the group of flasks had been flamed, the throat of the first one had cooled sufficiently to allow handling. The clot was loosened from the floor of the flask by means of the spatula (fig. 2, *B*); and after a very light flaming of the throat which did not heat the glass through, the clot was slid onto the platform of the dissecting dish assembly, which consisted of a covered pyrex petri dish, 15 mm. high and 100 mm. in diameter, with half of a

75-by-10-mm. dish (either top or bottom) placed in it to form a platform just below the surface of the lid of the pyrex dish. Dissections were made on this platform, and unnecessary fragments of clot and waste fluid were pushed over the edge into the surrounding moat. Each individual culture was dissected separately, a heavy dissecting needle and a No. 3 or 5 Graefe cataract knife being used. Separate dissecting dishes and instruments were used with each culture. The heavy ridge at the edge of the clot and all parts of the clot containing no cells or too few cells for satisfactory transfer were cut away. From the remainder, as many strips as desired were cut.

The size of the explants varied somewhat. In the first few generations an attempt was made to get strips about 3 mm. wide and 15 mm. long. However, better results were obtained with strips having a somewhat greater width, and in the later work an attempt was made to get them 4 to 5 mm. wide and approximately 20 mm. long. Usually three or four strips were cut at right angles across the original explant. In some instances only two strips, lying parallel, and just lateral to the old explant, were cut, and in others diagonally cut strips were used. The exact type of explant was so chosen as to give what seemed the best possible chance of growth from the old sheet of cells.

The explants were handled rapidly to avoid their drying out; as soon as they were cut, they were placed in approximately 0.7 cc. of saline solution in one of the depressions of a spot plate (Corning No. 7220) set in a 150-by-20-mm. flat pyrex preparation dish. The adhesive label of the flask was transferred to the top of the dish to mark the culture, while the order of explants was identical with that on the work sheet. Each strain of cultures was kept in a separate depression plate so that there was no chance of confusing individual cultures or different strains. Each culture was labeled and records were kept on the origin of each subculture.

Preparation of the Clot

From 0.4 to 0.5 cc. of chicken plasma was inserted in each Carrel flask from a 5-cc. pipette (figs. 2, *C*, and 3). In the early part of the experiment 0.4 cc. was used, but this gave a clot which in some instances was so soft that holes developed near the end of the transfer interval. When the amount was increased to 0.5 cc., no further trouble was experienced. The plasma was shaken over the entire surface of the floor of each flask, and 0.7 to 0.8 cc. of a mixture of 40 percent horse serum, 20 percent chick-embryo extract, and

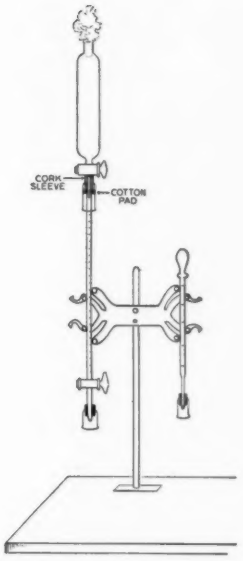

Figure 3.—Pipette and burette unit for planting cultures. The rubber bulb on this pipette is of 5-cc. capacity. For washing cultures and adding fresh culture fluid, a second burette unit was substituted for the pipette.

40 percent saline was then added. The explant was inserted in the flask at once by means of the spatula, flattened out, and centered. Each flask was closed with a rubber stopper, marked with a previously prepared adhesive label, then leveled until the mixture had formed a firm clot, and racked. Later all flasks were transferred to the rocking shelves in the incubator. Twenty-four hours later, 1 cc. of a mixture of the horse serum, embryo extract, and saline in the foregoing proportions was added. For cultures that were to receive carcinogen, it was contained in the horse serum in such concentration that each cubic centimeter of the final supernatant culture fluid contained approximately 1γ of carcinogen.

Washing and Renewal of Fluid Culture Medium

The fluid culture medium was changed three times weekly, usually on Monday, Wednesday, and Friday. The only serious deviation from this was near the end of December 1941 and in early January 1942, when the change was made only every 3 or 4 days. The resultant injury to the

548963—43——4

normal cultures was severe and was reflected in the noticeably poorer growth for nearly three generations.

In changing the fluid medium, the flasks were wiped off with a towel moistened with absolute alcohol and the cultures arranged on the table in numerical sequence within each set; the order of the sets was as follows: (1) Cultures which had never been subjected to carcinogen (all flasks carried white labels); (2) cultures which had at some time been subjected to carcinogen but were no longer (flasks carried green labels), the various sets being arranged in order so that those most recently removed from the carcinogen were always handled last; and (3) cultures which were being carried in carcinogen (flasks carried red label) and which were always handled last of all.

Aseptic precautions were taken and all normal solutions made up before any carcinogen-containing solutions were opened, and the culture fluids were correctly mixed in the separatory funnels of the burette units (fig. 3). Each funnel was then mounted on its burette which was locked in the burette rack. The other half of the rack carried a similar unit filled with saline solution.

The throat of each flask was flamed, the rubber stopper slipped out, and the end of the throat again flamed very hot. The old fluid was sucked out with a sterile glass needle attached to a fluid reservoir and a source of vacuum (fig. 4). With the burette arrangement, 3 cc. of saline solution was run into the flask, which was lightly stoppered with a sterile cork, and set aside, until each flask in the set had received saline. The suction needle was sterilized with heat after each use. After the last flask in the set had received saline, the first one was uncorked, the neck lightly flamed, the saline sucked out, 1 cc. of culture fluid added, and the flask at once sealed.

The burettes (fig. 3) were of 25-cc. capacity, graduated to 0.1 cc., and sturdily built to take relatively rough handling. The tip of each was protected, while in use, with a short glass sleeve held on by a cork collar. The open end of the sleeve was flared slightly for easy insertion of the throat of the flask which could be slipped in a distance of 7 to 8 mm., while the burette tip projected through the cork collar about 3 or 4 mm. The sleeve fit rather snugly around the throat of the flask. The freshly flamed throat inserted into the sleeve was free from bacteria, shielded so that bacteria could not fall into it, and so centered around the burette tip that the latter never touched the inside of the throat. In this way the likelihood of spreading bacterial contamination through consecutive flasks was obviated.

A wad of cotton in the upper end of the sleeve permitted air to flow into the burette as fluid was run in and withdrawn. All stopcocks were lubricated with sterile (autoclaved) heavy petroleum jelly. At the end of each run the equipment was disassembled, the cork collars were destroyed, and the glass parts of the apparatus were cleaned (with

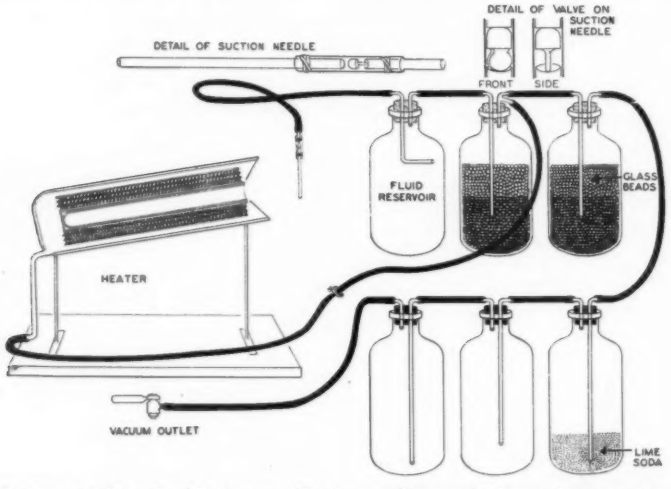

FIGURE 4.—Suction needle unit with heater. The heater diagram is drawn disproportionately large to show detail. Details of suction needle and valve assemblage are also enlarged.

acid if the unit had been used with culture flasks that contained or had ever contained carcinogen).

The suction needle (fig. 4), a piece of standard wall pyrex tubing 6 mm. in outside diameter and 150 mm. long, was attached to a length of gum rubber tubing 60 mm. long, with 6-mm. bore (1.5-mm. wall thickness). A glass bead of special design was inserted midway the length of the tubing to serve as a valve. When the outside of the rubber tube was squeezed gently with the fingers, the resulting deformation caused a leakage of air around the bead. The tubing was sufficiently soft and pliable that a relatively gentle pressure near the bead gave a vigorous suction at the tip of the glass needle. Figure 4 shows in detail the setup. The bottles containing the glass beads each had a 1½-inch layer of sulfuric acid in the bottom, which served to trap any possible spray containing traces of carcinogen that might be carried over into the house vacuum line. The sulfuric acid was replaced about every 6 to 8 weeks. The first of the three bottles connecting with the vacuum outlet contained lime-soda to neutralize any acid spray carried to it.

A small electric heater to sterilize the suction needle between uses is shown in figure 4. The heating element was wound around a 15-mm. (inner diameter) metal tube about 6 inches long and insulated from it with several layers of asbestos paper. The tube was sloped so that the needle could be rapidly inserted without danger of its sliding out. A small glass test tube was used as a lining to protect the metal tube from corrosion and to prevent the carrying over of metal oxides into the culture flasks. It was changed at the end of each series of cultures.

The winding was so designed that, within a few minutes after being switched on, the heater reached and maintained a temperature of about 340° C. within the lumen of the glass tube, as measured by a thermocouple, while a suction needle containing several drops of water boiled dry within 7 seconds after insertion into the heater tube. Inasmuch as the interval between using the suction needle on two consecutive flasks was about 20 to 30 seconds, the hazard of carrying a bacterial infection from one flask to the next was avoided.

Figure 4 shows the metal casing projecting over the open end of the heater, and also the hose connection with the acid shield on the vacuum line. Through this line a slight current of air was kept flowing over the front outlet of the heater in order that any traces of fumes liberated from the heated needle might be carried into the sulfuric acid trap and not released in the room.

When this unit was originally designed, the question arose whether it could be used safely on the cultures lest the hot needle injure them by its own heat or by fluid heated to boiling and expelled from it onto the sheet of cells. The system finally worked out has now been used for more than 2 years and has given satisfactory results. To remove fluid from a culture flask, the needle was removed from the heater and at once inserted into the open mouth of the flask. Before it entered, however, the operator had already exerted pressure on the rubber tubing around the glass-bead valve and so established a rapid flow of air through the glass tube. The tube was lowered along the inner bottom surface of the flask throat; meanwhile an uninterrupted flow of air was maintained through the needle. As the needle tip reached the floor of the flask, the latter was tilted very slightly but not so much that fluid ran into the open end of the throat. The fluid was sucked out cleanly and in a fraction of a second. The needle was removed, the pressure on the valve released, and the needle returned to the heater. In this way a needle could be used for evacuating a series of 150 flasks without clogging. If the needle clogged after a short time or if it did not suck out the fluid cleanly and almost instantaneously, it was not being correctly manipulated. Usually in such cases the valve was not open sufficiently. Once an operator was trained to use this device, the average time necessary to handle each flask was approximately 60 seconds. Cultures used with this unit have never shown local injury to cells near the throat of the flask, and only a very few have had to be discarded because of some incorrect behavior of the suction device.

After use, the suction-needle equipment and its fluid reservoir were disassembled. Rubber units were destroyed, and glass units given the usual acid treatment. The tip of the glass inlet tube of the first acid bottle on the suction unit was flamed very hot to eliminate any traces of carcinogen that might have adhered from spray passing through it, and it was capped until the next change of fluid. The aluminum front of the electric heater was heated with an open flame as hot as possible without fusing to destroy any traces of methylcholanthrene that might have been left on it by the needle.

No culture that was receiving carcinogen or that had ever received it was ever opened until all normal cultures had been washed, fresh fluid added, and the cultures sealed. Similarly, no culture that was receiving carcinogen was opened until those that had once had carcinogen but were no longer receiving it were resealed. There was no exception to this rule. Careful checking and

the different colored labels on the three series precluded any chance disarrangement in the order of handling.

In a few instances when one or two drops of a carcinogen-containing solution were accidentally spilled on the table or the floor during the handling of the cultures, the solution was cleaned up at once by wiping the area repeatedly with a succession of cotton swabs soaked in acetone or benzene.

Procedure for Recording Changes in Size of Cultures

One of the usual techniques, and the simplest, for estimating the enlargement of a culture is to determine and plot the area at different times after planting. Parker (7) and Cunningham and Kirk (8), among others, have pointed out that this is not a true index of growth but that it is complicated by other factors. When cells are treated with methylcholanthrene, the number of cells in each unit area of the treated cultures is different from that in the controls; the curves obtained are, therefore, certainly not a reliable index of growth.

Even though this be true, the need was felt for some simple and easily applied index which could be used to keep some record, other than that of cell description, of the deviation of the carcinogen-treated cultures from the normal. Since the earlier work had shown that the action of the carcinogen could be detected within a very short time by the lagging of the rate of increase in area or width of the treated cultures and since this retardation seemed to be associated with the action of the carcinogen, it was felt that this index was worth using as a working guide. The index, as in the previous work with strip-shaped explants (2), consisted of a record of the changes in the average width of a culture, the length of the strip being left out of consideration. In all instances the width was determined by measuring the culture under a compound binocular dissecting microscope fitted with paired 1.0 × objectives and 9 × oculars, one ocular carrying a special micrometer disk divided, as shown in figure 5, to read numbered intervals of 1.0 mm. actual size of the object, with ruled subdivisions of 0.2 mm. This gave a simple and rapid means of estimating the width of the cultures to 0.1 mm. without subjecting them to intense light. The data obtained by this method of measuring were collected on all cultures and plotted to give curves for each generation of each culture strain. These curves are not presented in this paper on account of the difficulties in reproducing them for publication.

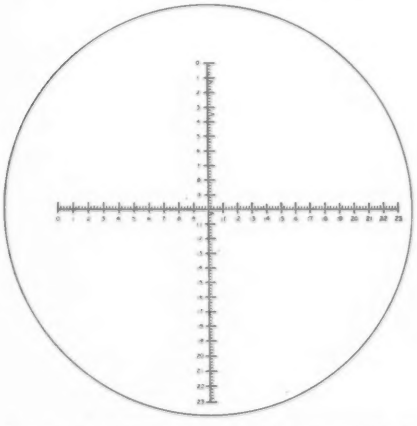

Figure 5.—Ruling of ocular micrometer disk for recording changes in the width of cultures. The ruling is displaced slightly from the center to facilitate the measuring of small cultures.

The interruption of these curves because of periodic transplantation of the cultures into fresh flasks offered a serious difficulty to obtaining a satisfactorily connected picture in the culture strains under experiment. To show this progressive change, the width of the zone of new growth was taken at 5, 10, and 15 days after explantation. These three readings were averaged and plotted as an average width at a time 10 days after explantation (fig. 6). In a few instances where the width curves ran only 13 or 14 days, the value for 15 days was obtained by extrapolation. These various average points on any one strain of cultures were then joined by a continuous line. In this way, for any one carcinogen-treated strain one continuous line was obtained, and for its normal control cultures another line was similarly obtained. The number of cultures averaged to give each point on the curve is shown by the number opposite the point, while the letter adjacent indicates the cell strain used to give that point. This notation is necessary since often one set of controls was used for more than one experimental series. To show the relationship of control and experimental culture when this occurred, the curve from the control was repeated for each series for which it was used. Similarly, where strain N, for instance, originated from strain O, the early part of the curve from strain O has been drawn in for the strain N curve to complete the picture of the curve. The time each experimental strain was left in the carcinogen is

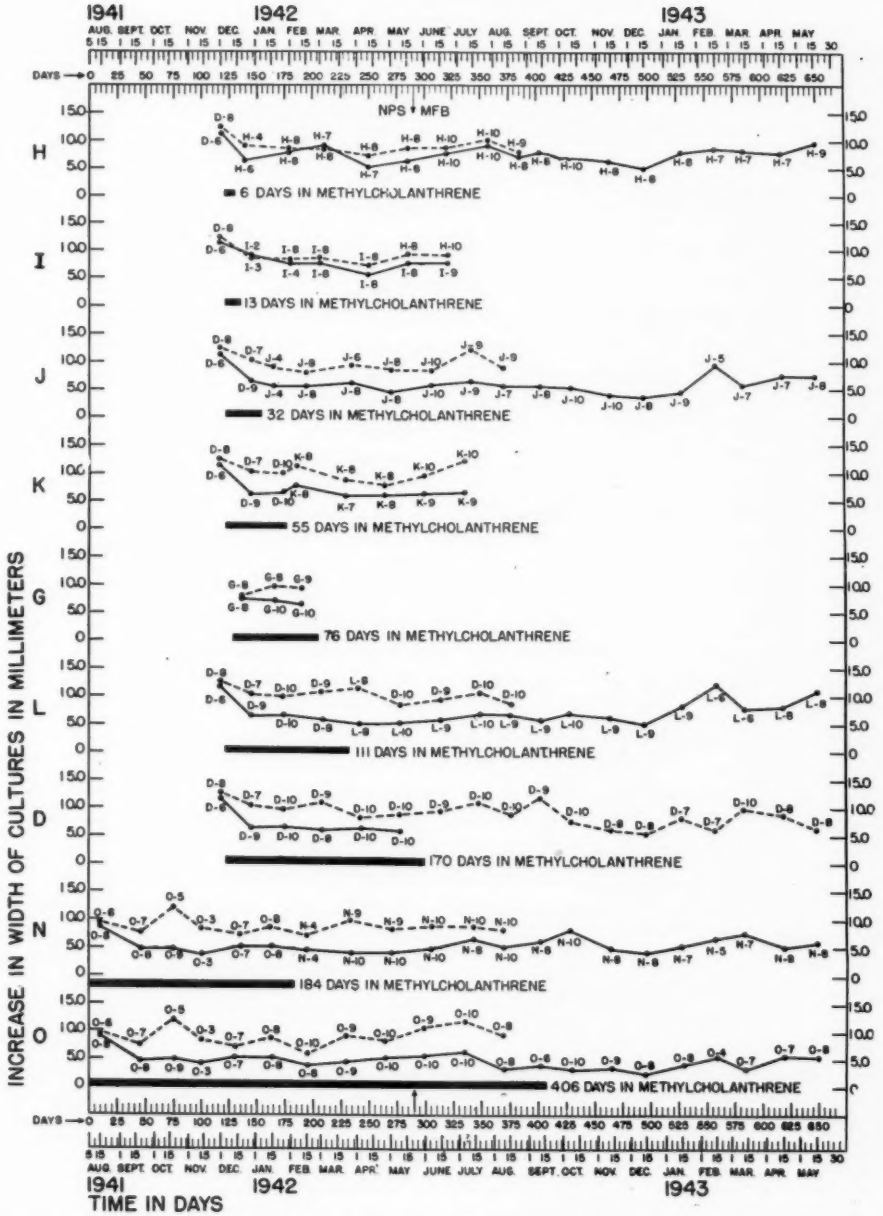

FIGURE 6.—Continuous width curves for all strains. The carcinogen-treated cultures are shown by the solid line; the control is shown by the broken line. The time the treated cultures were left in carcinogen is shown by the heavy black line. Each curve is identified according to its strain of cells by the large letters opposite it.

shown by the length of the heavy black line below the respective curves. These continuous width curves are presented through May 18, 1943.

This type of curve was plotted for each of the strains studied. The curves (fig. 6) are designated continuous width curves.

RESULTS

Origin and Description of the Primary Normal-Cell Strain

The strain of fibroblastlike cells used was originally taken from a 100-day-old male mouse of the C3H strain, Andervont substrain. On October 18, 1940, the mouse was decapitated. Without including muscle tissue and the regional lymph node, the connective and fatty tissue pad along the side and just in front of one of the hind legs was removed and cut into six strips each about 2.2 mm. wide and 6.0 mm. long. Each strip was planted in a separate flask. When examined just after planting, much of the tissue was seen to consist of fat cells. Five days later, the only sign of migration was an occasional projecting cell. At 10 days, the width of the strips had increased from 2.2 to 10.2 mm., and the growth was recorded as luxuriant and even but somewhat loose; there were numerous cells in the growth zone which contained large fat droplets, and it appeared certain that the fat cells had contributed materially to this zone. From the reduction in size of the fat droplets, it also appeared that many of these cells were losing their fat droplets. Growth continued until 42 days after explantation. At this time, one of the cultures had been lost through accident. The remaining cultures showed an average width of 18.0 mm. The fringe of cells showed even less fat than earlier, because of a reduction both in the number of cells containing fat droplets and in the size of the contained droplets.

The cultures were transplanted into from 5 to 8 flasks each, the explants being selected so as to include none of the original explant. All grew luxuriantly, and all except 1 were carried on for a number of generations and then closed out. This 1 was transferred to 3 flasks 63 days after planting. In this third generation growth was excellent and even. One selected culture was transferred to 6 flasks 43 days later. One of these was again transferred to 10 flasks 39 days later, and of these 1 was transferred to 5 flasks 30 days later (May 22, 1941). Of these 5 cultures, 2 were selected and built up into a series which was used for the experiments reported herein. All cultures used in this work originated from 1 of these 2 cultures.

The amount of fat droplets seen in the cultures had rapidly lessened until by the third generation often whole cultures were entirely free from all except the few very small fat droplets normal to growing fibroblasts in this culture medium. When the cultures were carried in the same flask so long that the density of the cell population caused overcrowding, particularly among cells deep within the clot, some cultures showed a rapid temporary formation of large fat droplets within the cells, but this formation subsided at once when the cultures were transferred to fresh flasks. In the later history of the strain, large fat droplets showed up sporadically under these conditions, but the number of cells showing such droplets was small in relation to the number of cells in the culture. The droplets disappeared as soon as the culture was shifted into a fresh flask with fresh culture medium.

The structure of these cultures was normal for cultures of fibroblasts previously described (*2*), the architecture was loose, and the cells were of characteristic shape, adhering to each other usually by terminal processes. Figures 8, *B*, 9, *B*, and 11, *B*, show typical culture structure. The refractive index of the cells was low,

and particularly in very dense cultures the cells were often hard to see. There was no clear relationship between relative growth on the surface of the clot closest to the fluid and that nearest the glass in the early life of the cultures. At both surfaces the cells seemed to be rather similar, although at each they were usually more flattened than were those within the body of the fibrin clot.

The rate of growth of the cells was comparable with that of earlier strains of normal fibroblasts carried in this culture fluid. There was variation from flask to flask, and growth during some generations was better than during others. Frequently these variations could be traced to minor differences in handling; too frequent shifting of the cultures to a new generation resulted in smaller explants and in low cell density within the new explants, and these poorer explants in turn gave rise to even poorer growth. Similarly the holding of cultures too long before transfer resulted in diffuse central necrosis and poorer growth when the cultures were transferred. The best interval for transfer with the range of explants used was probably about 35 to 40 days.

Sequence of Changes in Freshly Transplanted Normal Cultures

During any one generation the sequence of changes in these normal cultures was approximately as follows:

For about 8 to 12 hours after explantation there was no appreciable sign of migration of the cells. At 24 hours there were often a few cells or cell processes out, which lay at all levels within the thickness of the clot and often resembled spikes. This fringe got wider and denser; at several days there was a luxuriant growth, the cells of which usually showed a definite tendency to concentrate along the interfaces of the clot, that is, in the layer of clot closely adherent to the glass and the one next to the overlying fluid. These layers will be referred to later as glass and fluid interface layers, respectively. The cells at the edge of the migrated zone were typically fibroblastlike, with characteristic spindle, flattened-spindle, or less frequently triangular shapes, and with frequent, long, terminal, threadlike processes. At this stage the culture was often troublesome to photograph with higher power lenses for several reasons: (1) There were irregularities in the thickness of the clot very close to the explant; (2) in reaching the glass and fluid interfaces, the cells within the clot sometimes lay along a slope so that when examined with higher lenses the whole length of the cell could not be focused at once; and (3) the layer of cells at this stage was frequently very thick, often with a lack of areas of cell density suitable for photographic records. Nor was the comparison of the increase in size of the clump with that of carcinogen-treated cultures as satisfactory during the first few days as it was a little later, since the experimental and control cultures had not grown sufficiently long to indicate clearly divergent rates of growth.

As the growth of the culture continued, there was a stage at about 8 to 10 days which was probably the most useful for comparing different cultures. The cells were not laterally coherent as the growth of the cultures was typically loose, mitoses were frequent, and there was no sign of degeneration. At this time no regularity was observed, whether the glass or fluid layer was the larger, although as a rule the edges of the fluid layer were somewhat looser. The edge of the normal culture was usually not too loose to permit the estimating of the culture diameter. At this stage also, experimental cultures had had time for their width curves to develop any divergences.

In the later growth of the culture, a number of complications interfered with the determination of the rate of increase of the size of the clump. Measurements of the width of the normal culture were often complicated by the extreme looseness of the sheet of cells. In some instances, there was no real edge to the culture, loose cells reaching the edge of the flask at 18 days of culture growth. This condition was worse at the fluid interface where sometimes the lack of a definite culture edge was caused by the fact that the cells had been washed loose from the culture and had reanchored themselves in the more peripheral parts of the clot. Often, too, little clumps of cells that had broken loose from the culture at its planting proliferated and at this stage reached such a size as to interfere with determinations of the size of the central clump. Consequently, width curves were irregular in this later period. Frequently the cell sheet at the fluid interface had grown so large and dense that study of the cells at the glass interface was obscured. With a very dense and rapidly growing clump, the study was further confused by traces of diffuse necrosis in the central area of the glass layer.

Once the loose cells reached the edge of the flask, growth could obviously not increase the diameter of the culture. Instead, from about 21 to 30 days on, the growth of the culture was manifested as increased cell density in the more peripheral areas. This increase tended to fill in the body of the clot. The culture was more difficult to examine and to photograph. In its last stages diffuse necrosis began to appear, first along the central area of the glass interface. When a large strip explant was used, the culture in this interval usually thickened to a heavy sheet of cells that reached the edge of the flask. With smaller explants, this migration to the edge of the flask was slower.

The foregoing general description held approximately for normal stock cultures and for controls up to the time lateral cohesion of the cells was observed. Owing to the various complications that occur during the very early and the very late life

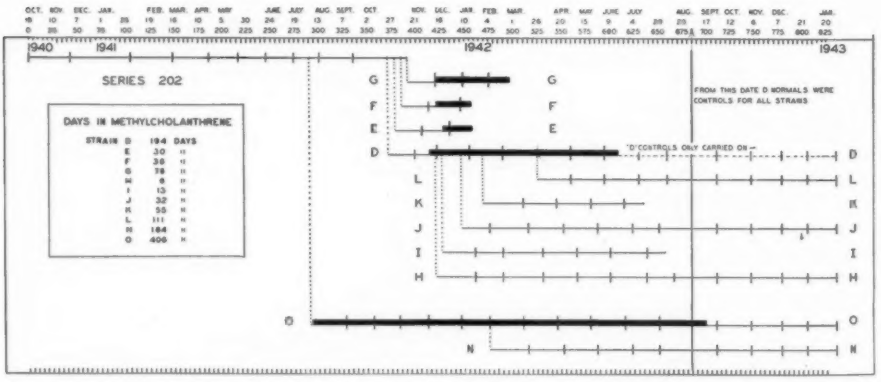

FIGURE 7.—General relationship of the culture strains. The interval during which any strain of cells received carcinogen is shown by the very heavy lines, while the time at which the strain was removed from the carcinogen is shown by the ending of the heavy line or by the vertical dotted lines which indicate when the culture was lifted out as a separate strain. Approximate times of changes of the cultures into new flasks, with corresponding changes of the fibrin clot, are shown by the short upright lines arranged along the horizontal lines. The letters denoting the different cell strains correspond to the strain labels in our records and are used to identify them.

of the cultures, in these experiments an attempt was made to correlate visual, photographic, cinematographic, and stained-slide records of the cells within the interval from 8 to 15 days after the culture was planted, preferably 8 to 12 days after. At this time, there were usually satisfactory areas of cells clearly defined along the glass and fluid interfaces, the sheets were not too dense for examination, and there was no necrosis of the normal cultures. Furthermore, as will be seen later, this period was probably the most suitable one for study of carcinogen-treated cultures.

Relationship of the Experimental Cell Strains

The relationship of the different strains of the carcinogen-treated cultures may be seen from figure 7. Until August 1942 each strain of cells was carried with its own control. All cultures were in the same culture fluid medium; but since not all the cultures could be transferred to fresh flasks simultaneously, different strains with their individual controls were transferred on different days. In order to compare the different strains more accurately, in August and early September 1942 the strains were regrouped. Since all control cultures had a common origin and since recognized morphologic differences between them were extremely slight, the controls of strain D were carried on as the control on all cultures and all other control strains were closed out. The carcinogen-treated cultures were then regrouped into several sets, so that the cultures of each set were transferred on the same day with the same solutions and so that each set contained cultures from strain D control, and H, J, L, N, and O carcinogen-treated strains. This reorganization allowed close comparison of the different strains under identical conditions.

In the following description of the changes in the cells of the different strains, the progress of the continuous width curves is given to the end of May 1943 and the cell changes observed in the living cultures to September 1942, whereas the cell changes from October 1942 through May 1943 are given in another section, in which the cells of the different strains are more closely compared.

Comparison of Continuous Width Curves for the Different Strains Through April 1943, and Description of Cell Changes Observed in the Cultures Through September 1942

Strains N and O (Series 208)

Strain O, the first of the experimental strains, was started August 5, 1941, after the cells had been carried 291 days in vitro. Four cultures were selected, and from each, two strips were cut lengthwise along the explants. Each was in turn halved transversely. Two explants from each original culture were maintained as controls; 24 hours later the other two explants from each culture were started in the methylcholanthrene solution. Two of these carcinogen-treated explants were damaged with a hot spatula and were discarded shortly after planting. The strain has been carried on by consecutive transfers up to the present time. The continuous width curve of this series is shown in figure 6, section O.

During the early days of this strain, there was no evidence that the carcinogen caused any accelerated rate of increase of width of the cultures. Six to eleven days after the addition of the carcinogen, the width curve of the treated cultures showed a definite depression relative to that of the controls. At 14 days, the depression was more severe; in the second generation it was even more so.

A visual examination of the cells during

the first few days after addition of the carcinogen showed no clearly defined change. There was no sign of lateral cohesion or of ribbon or sheet formation. If there was any degeneration in the cultures, it was so slight that its existence was uncertain. The only suggestion of an injurious influence was some slight lateral shrinkage of some of the cells.

At 45 to 48 days after addition of the carcinogen and 11 to 14 days after transfer, the treated cells were less spindle-shaped, more sheetlike, with definite shortening of their slender terminal processes. The cultures were more compact than normally, and their edges appeared less reticular and loose and showed fewer loose cells. There was a definitely increased irregularity of cell size. Numerous mitoses were seen in both experimental and control cultures.

At 52 days, 17 days after transfer, the changes described had progressed. The cells were very granular; some cells were up to eight times their normal size; numerous cells appeared in mitosis in both experimental and control cultures.

At 73 to 76 days, 9 to 12 days after transfer, the terminal processes of the cells were shorter, more blunted, and showed definite lateral irregularities suggestive of amoeboid rippling or frothing. The cells were still spindle-shaped and adherent laterally so that the edge of the culture appeared even more compact and less reticular or lacelike in architecture (compare fig. 8, *A* and *B*). There were few, if any, loose cells. The glass interface layer was dominant, and there was little or no fluid layer at this stage.

At 97 days, 7 days after planting, the glass interface layer was extremely compact, with few or no loose cells at its edges. The fluid interface layer was less pronounced and less characteristically coherent than was the glass layer. The culture edge showed numerous short cell processes, and there were only occasional epitheliallike lobes at the edges. The terminal processes of the cells were usually very short, and the amoeboid structure of the lateral edge of these processes had extended farther toward the mid region of the cell. The whole lateral edge of the processes often had an amoeboid appearance (cf. fig. 9, *A* and *B*), which was so pronounced that in some instances it was hard to delimit the exact edge of the cell. The cells seemed more granular than the controls, and there was more local cell disintegration.

At 106 days after addition of the methylcholanthrene and 16 days after planting, the cultures were very dense, and there was a large area of central necrosis. The cells were very closely coherent into sheets over extensive areas, and the culture edge was abrupt (figs. 10, *A* and *B*, and 11, *A*). The cells shown in figure 11, *B*, are the control for those in figure 11, *A*.

In some instances the edge was made up of short projecting spikes of cells, in others the cells were flattened, often epitheliallike. The cell processes were extremely short, while the amoeboid rippling or bubbling along the edges of the cells often extended the whole length of the cells. There was extensive cytoplasmic granulation.

During the subsequent interval up to 184 days after the first addition of carcinogen, there was no feature of note except a gradual progression of the processes already described. Often in this period the cultures showed lobed edges typical of epithelium in culture.

On February 6, 1942, after 184 days in the carcinogen and 31 days after planting, the cultures had gone through six generations of transplantation. At this time a group of four cultures was removed from the carcinogen and set up as strain N (fig. 7). From this time on the cultures of

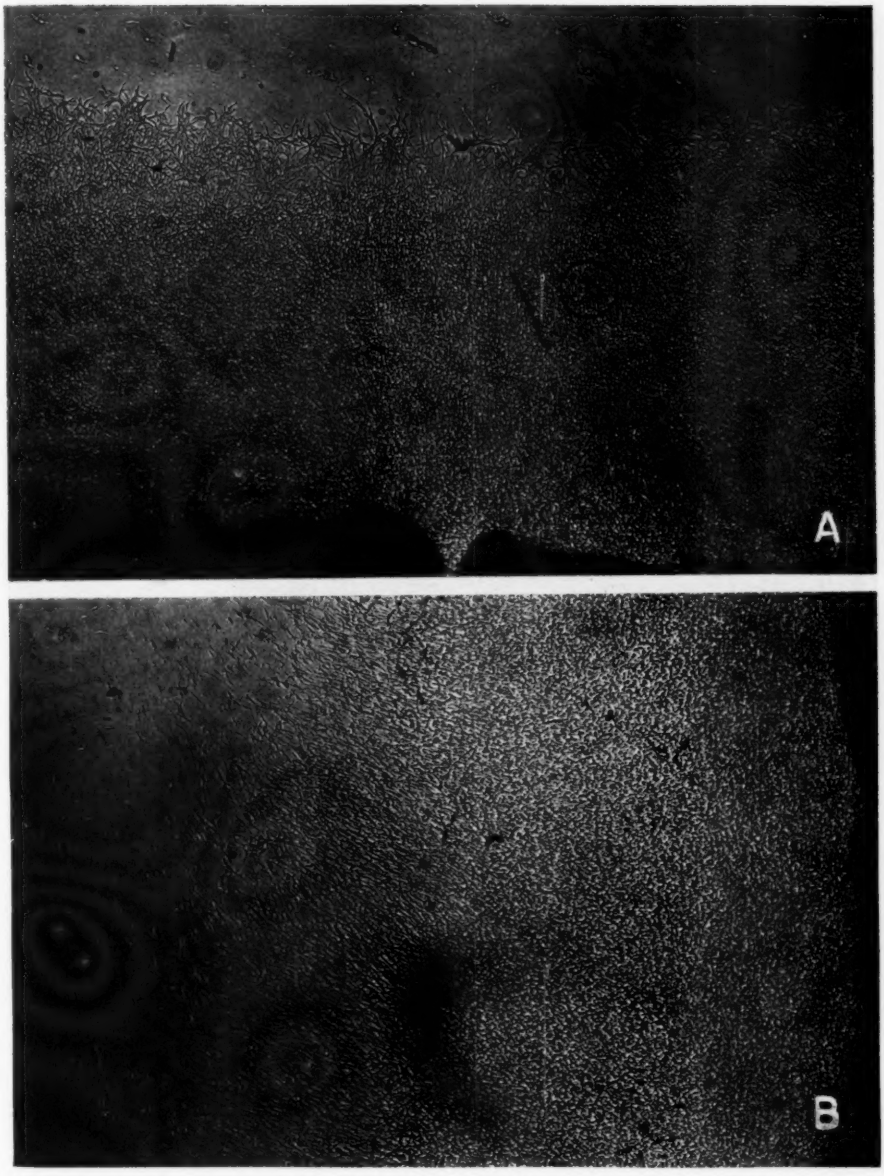

FIGURE 8.—*A*, Culture from strain O, 76 days after first adding carcinogen and 12 days after planting. The black circle is an optical imperfection; *B*, Normal control cells. Both *A* and *B* are cells at the glass interface. × 20.

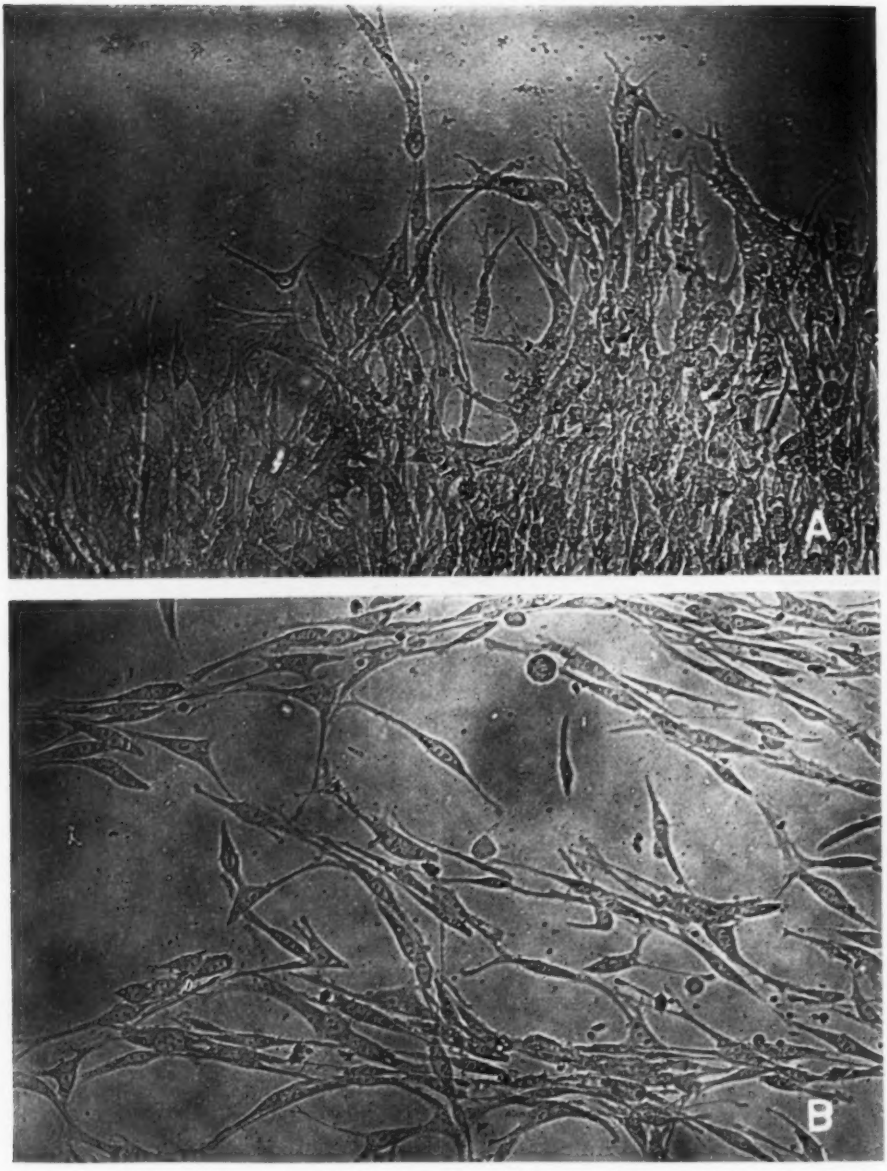

FIGURE 9.—*A*, Edge of culture of strain O, 97 days after addition of carcinogen and 7 days after planting. Note shortening of cell processes and occurrence of lateral frothing or rippling; *B*, Normal control cells. Both *A* and *B* are cells at the glass interface. × 200.

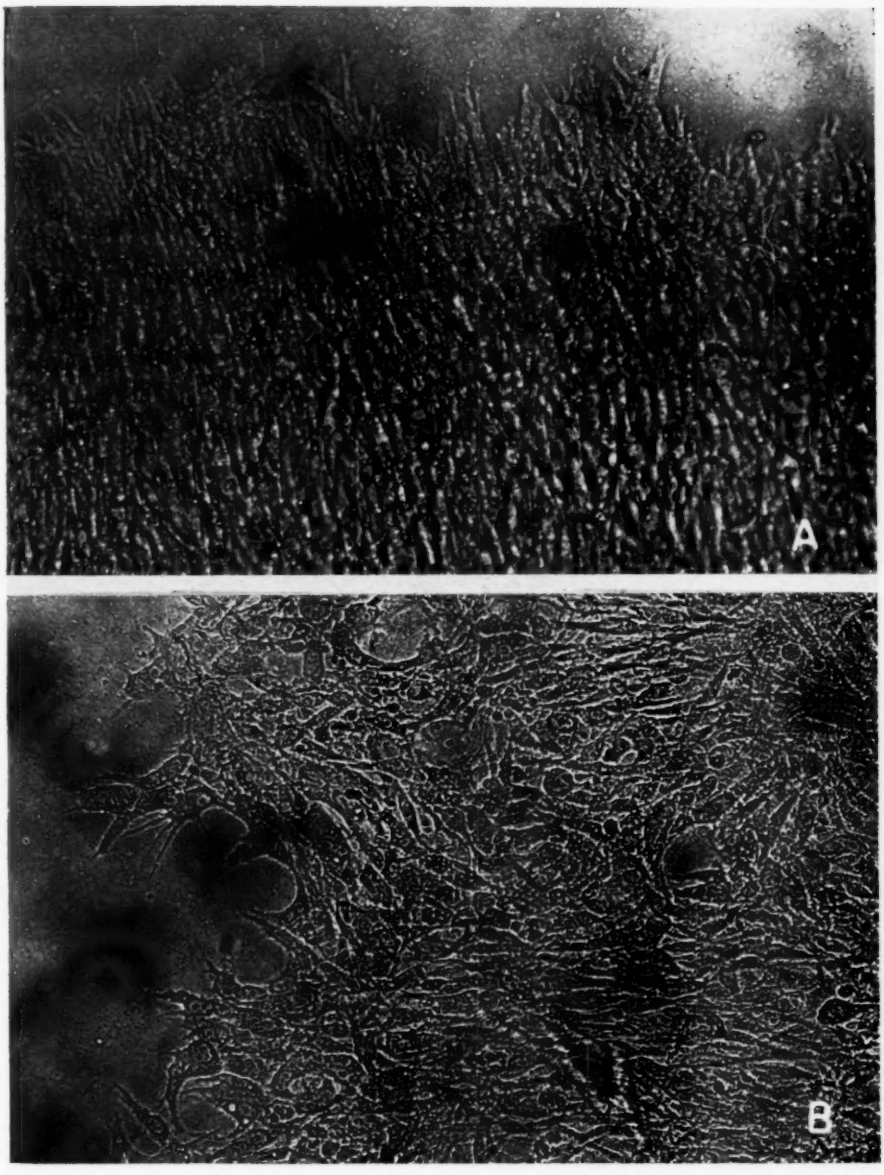

FIGURE 10, *A* and *B*.—Edge of culture of strain O, 106 days after addition of carcinogen and 16 days after planting. Note extremely short cell processes and definitely amoeboid appearing edges. Figure 9, *B*, can be taken as representative of the normal culture at this time. The dark areas of shadow in the print arise from droplets of fluid on the roof of the flask. × 200.

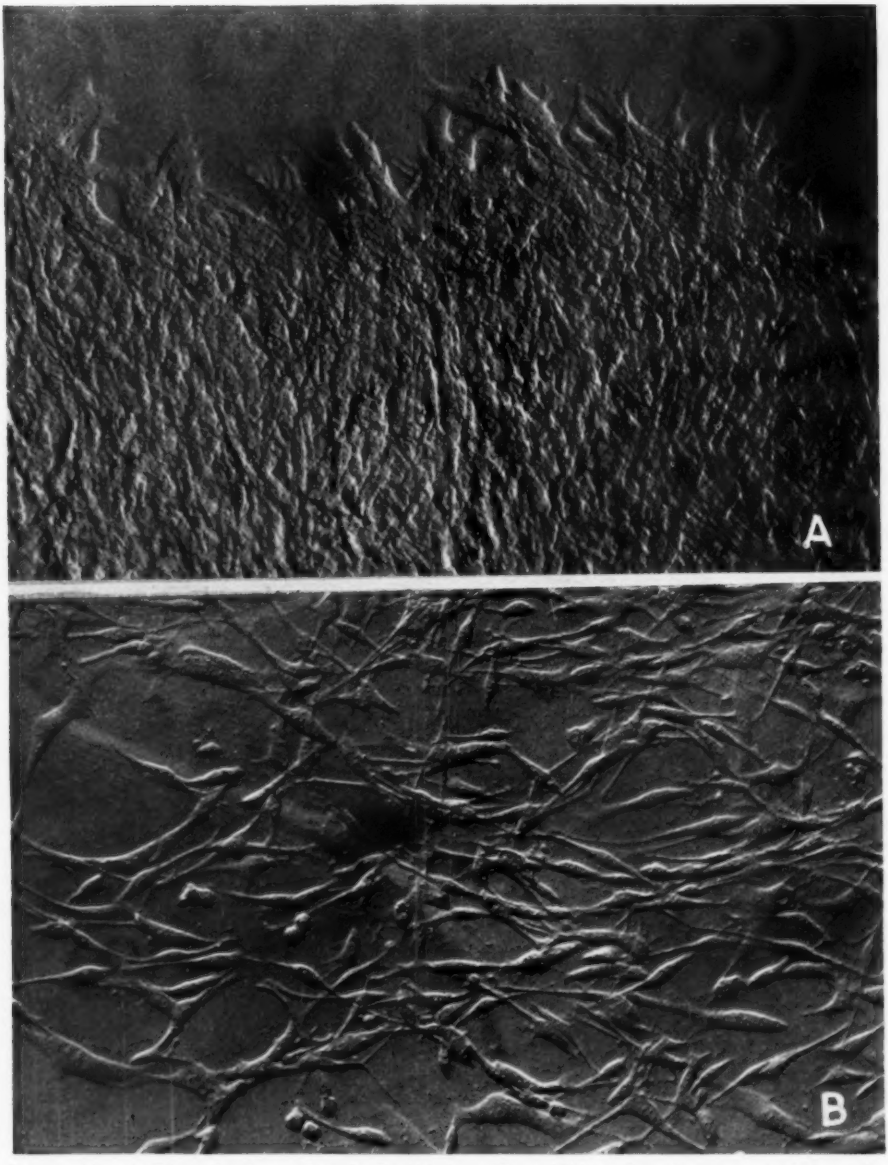

FIGURE 11.—*A*, Edge of culture of strain O, 132 days after addition of carcinogen and 12 days after planting. Note epitheliallike cell shape, the suggestion of granularity, the less clearly defined cell axis, and the shortened processes; *B*, Control cells. Both *A* and *B* are cells at the glass interface. Oblique lighting. × 200.

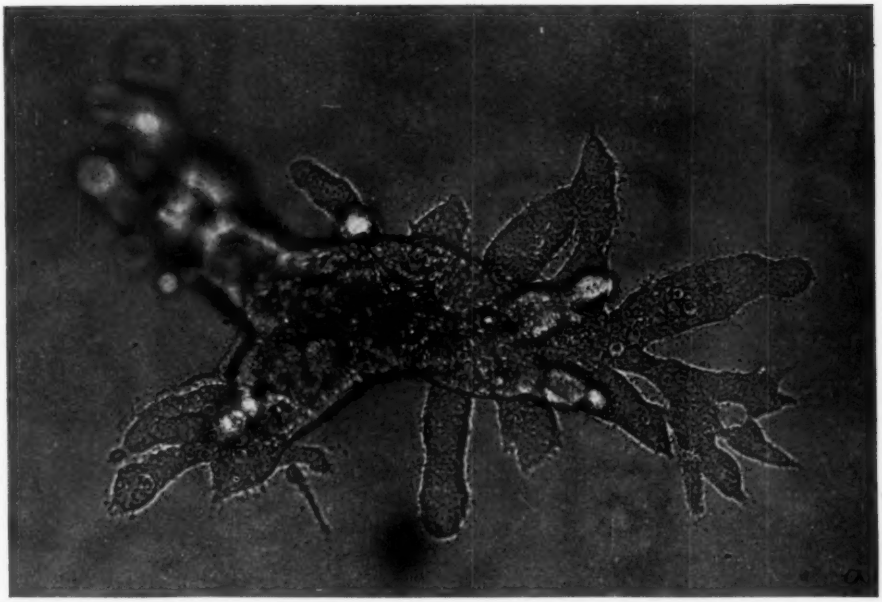

FIGURE 12.—An extremely small cell clump which arose from a fragment in a flask with a larger size clump of strain N, 57 days after removal from carcinogen and 18 days after planting. The clump is situated near and at the glass interface. One end of the clump is at a lower level and is out of focus. Note the close cell cohesion and the irregularity of the cell surface, also the complete absence of necrosis at a time when the central area of the larger clump showed extensive necrosis. × 200.

strain N were carried without further addition of carcinogen. Through the end of May 1943, the strain had been carried a total of 663 days since the original addition of carcinogen and 479 days since removal from it.

During the four transfers following the removal of this strain from the carcinogen, the continuous width curve (fig. 6) held to the same even, depressed level it had shown while the cells were in the carcinogen. With the omission of the first reading in the curve following the original addition of carcinogen, at which time the curve had not reached this low level, the average value for the curve through the fourth generation following removal from carcinogen was 4.6 mm. Beginning with the fifth generation after removal from carcinogen and including the 11 transfer generations through May 1943, the curve showed much greater fluctuation, while the average level of the curve for these 11 generations rose to 6.0 mm.

No great change in the appearance of the cells was noted in the period immediately following removal from the carcinogen. There was no evidence suggesting a further progression of the cell changes noted. The close coherence of the cells and the altered character of the cell surface at this stage are particularly well shown in figure 12, 57 days after removal from methylcholanthrene and 18 days after planting.

The strain of cells continued in the carcinogen after 184 days (strain O) was carried on uninterruptedly until September

16, 1942, or 406 days after the first addition of carcinogen. Following the initial depression in the first generation after addition of carcinogen, the continuous width curve of this strain held to a relatively constant depressed level through the eleventh transfer generation (fig. 6, sect. O). The average level of the curve during this time was 5.0 mm. Beginning with the twelfth transfer generation, there was a further depression of the curve which held to a relatively constant lower level for two generations.

At the end of that time so much difficulty was being experienced in transferring the strain (the average fringe of migrated cells was only 2.7 mm. wide at the end of 16 days, and even the more central cells of this fringe were already necrotic) that there seemed to be no doubt but that continuation of the cultures in the carcinogen would result in loss of the strain within the next two or three transfers. The carcinogen was therefore discontinued September 16, 1942, 406 days after it was first added to the cultures.

For the next three generations following removal of the culture from the carcinogen, the continuous width curve held to the same low level, the average level for these three generations being 3.0 mm. Beginning with the fourth generation after removal of the culture from the carcinogen, the curve showed increased fluctuation, while the average height of the curve during the next five generations (ending in May) was 5.0 mm.

The progression of cell changes in strain O during the period 184 through 406 days was a continuation of those shown earlier (fig. 13, *A* and *B*, and fig. 14). The adhesion became increasingly severe, with a further increase in production of dense sheets and masses of cells, the sheets at their edges often showing lobe-shaped projections similar to those seen in cultures of epithelial cells. The cells were short, often more or less rounded, and adhered laterally to those around them. Cell structure in such massive sheets was very hard to study; but where more isolated cells were found on the glass interface, the cells no longer showed the characteristic spindle shape of the fibroblast. The individual cells were usually irregular in shape, and there were often short lateral processes. The edges were often frothy and ruffled along the whole length of the cell (fig. 14). During the same period there was a further increase in the granulation of the cytoplasm so that the cells were extremely grandular. No well-defined alterations in the nuclei were observed in the living cells, although this point has not as yet been critically studied.

Series 202

Series 202 was planned to obtain more detailed data on the earlier stages of the action of methylcholanthrene, particularly the influence of graded doses. Strains D, E, F, and G were, therefore, started November 20 and 27 and December 4 and 11, 1941, respectively, while the carcinogen was added December 5, 19, 12, and 12. The addition of carcinogen to the series was so planned that in each of the strains it was made at a different time after planting, at 15, 22, 8, and 1 day, respectively.

Because of circumstances that had no connection with the behavior of the cultures, it was necessary to discontinue strains E, F, and G at 29, 36, and 75 days after the original addition of the carcinogen. For that reason, these strains contribute little to the data. The continuous width curve of strain G is shown in figure 6, section G. The early cellular description of these strains is similar to that of strain O.

Strain D, however, was continued and

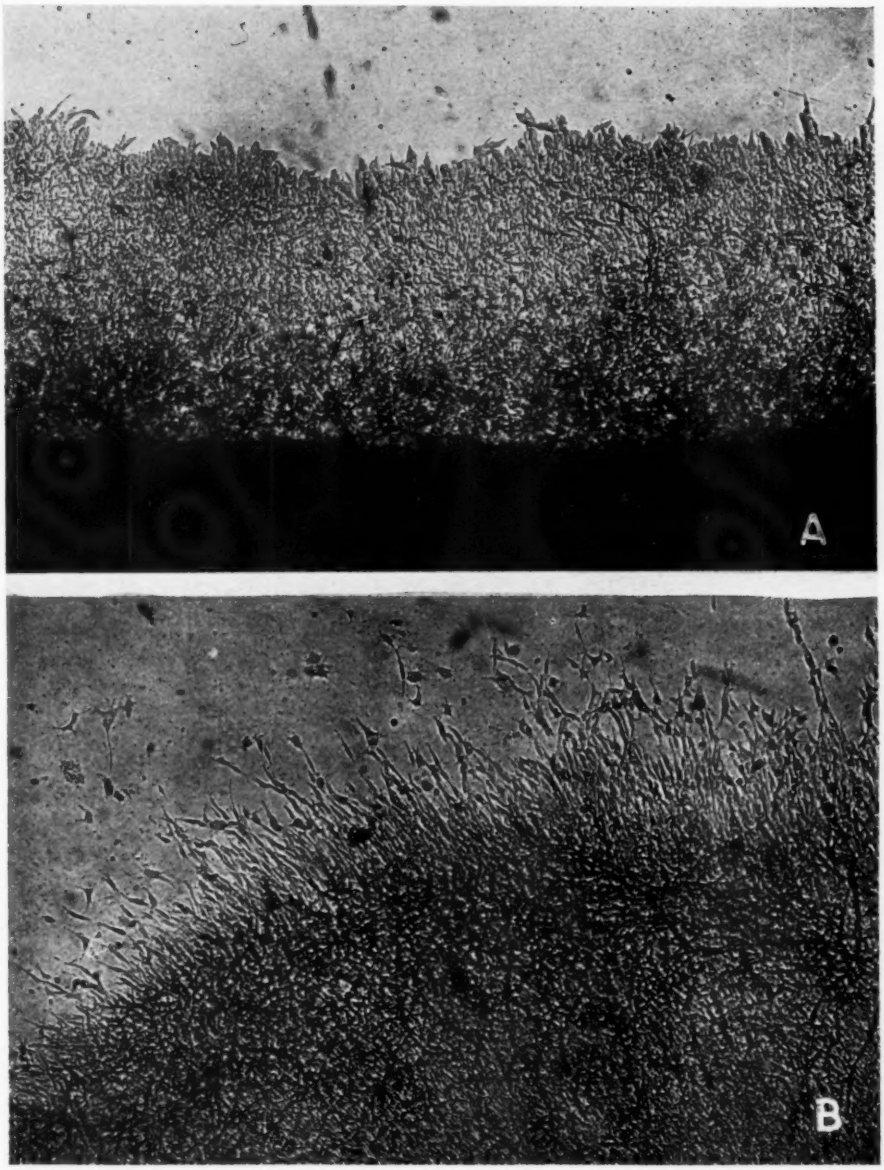

FIGURE 13.—Edge of culture of strain O, 403 days after addition of carcinogen and 11 days after planting. *A*, Cells of glass interface. Note the massive sheet formation and the very limited width of the zone of growth; *B*, Cells of the fluid interface. Note the relatively loose structure of the culture and the greater width of the zone of migrated cells Both *A* and *B*. × 40.

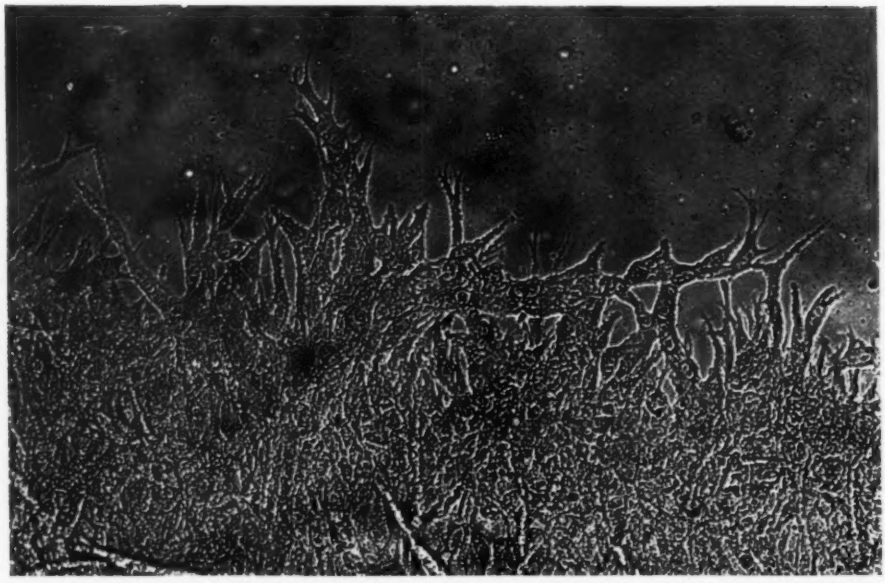

FIGURE 14.—Edge of culture strain O, 403 days after addition of carcinogen and 11 days after planting. Cells of glass interface, just before removal from carcinogen and at a time when growth was so slow that it seemed the series would be lost. × 200.

was studied in greater detail. The cultures of this strain were removed from the carcinogen at different intervals and were carried along with their respective controls. Strain H was removed at 6 days, strain I at 13, strain J at 32, strain K at 55, and strain L at 111 days after the first addition of carcinogen. With the removal of strain L from carcinogen, strain D experimental cultures were closed out, while the controls of this strain were run under their original label (D) as the control cultures for strain L.

Strain D.—The continuous width curves of this strain are shown in figure 6, section D. To get an uninterrupted record, these curves have also been included in the early part of the curves of strains H, I, J, K, and L.

The continuous width curve of strain D was depressed sharply in the second generation at 19 days after addition of the carcinogen. It is most likely that this depression could have been detected even earlier if the time of adding the carcinogen to the cultures had been different in relation to their last planting. In the four subsequent generations the strain was carried, the depression did not increase but growth was held to a level uniformly much lower than that of the control cultures. The average level of the curve for the second through the sixth generation was 6.3 mm.

In a group of four photographs each of controls and treated cells at 25 days after the first addition of carcinogen, one treated culture showed noticeable cell cohesion. There were very few loose cells. The cells were more sheetlike, with shorter and more amoeboid appearing terminal processes than the controls. In instances the cells adhered terminally to form strands and short ribbons of relatively uniform diameter. These formed loops and twisted into arches, occasionally joining other loops laterally. At 47 days, five of nine cultures photographed showed this cohesion. The cells at this time showed definite lateral amoeboid outlines of their terminal processes. The cohesion progressed at what appeared to be a rate approximately comparable with that in series 208, until the strain was closed at 194 days.

Strain H.—Strain H was removed from strain D after 6 days in the carcinogen, and its control was removed from the control of strain D at the same

time. When examined at this time and shortly thereafter, the carcinogen-treated cells looked entirely normal. There was certainly no sign of change of culture architecture, cell shape, or any increase in cell cohesion. In spite of this, the continuous width curves (fig. 6, sect. H) showed a sharp drop in the generation directly following the removal of the cells from the carcinogen. In the next generation, however, growth was practically equal to that of the controls; and in the generation following, the average width and continuous width curves were slightly higher than those of the controls. During the last part of the second generation after removal from the carcinogen, that is, 64 days after removal, only one culture of eight examined showed recognizable cohesion of the cells.

In the fourth generation following removal from the carcinogen, the continuous width curve of this strain showed a sharp drop, and at this same time nearly all the cultures of the strain showed definite cohesion of the cells. This general cohesion was at 131 days after the carcinogen was originally added and 125 days after it was discontinued. The edges of the cultures were abrupt, and there were few loose cells. The cells were spindle-shaped, shorter, more sheetlike than normal cells, and had short amoeboid-appearing terminal processes. Short cell strands arborescing and re-fusing were observed. There was an increased amoeboid appearance of the lateral edges of the cell, particularly along the terminal edges of the cell processes.

In the interval from the drop of the continuous width curve in the fourth generation to October 1, 1942, the series continued to show a consistent but limited cohesion of the cells with consequent alteration of the architecture of the clump. This cohesion never reached as severe a degree as that shown in either strain N or strain O.

While the continuous width curve of this strain has fluctuated probably more than any other experimental curve, subsequent to the depression in the fourth generation at the time of cellular cohesion and through May 1943, it showed no definite, recognizable trend of change. The average level of the curve after the removal of the cells from carcinogen and through May 1943 was 7.6 mm.

Strain I.—Strain I was removed from strain D after 13 days in the carcinogen, at which time a control was started from the control of strain D. As may be seen from the continuous width curve (fig. 6, sect. I), in the first generation after the removal from the carcinogen there was no depression noted relative to the controls. Since, however, this determination was made on only three flasks for the control and two for the carcinogen-treated cultures, it cannot be relied upon. Only in the third generation were there enough cultures to give a reliable average. At that time the continuous width curve was somewhat lower than that of its controls and continued so until the series was closed out July 28, 1942, 235 days after the original addition of carcinogen. The average level of the curve from the second generation on was 7.3 mm.

The typical cohesion noted for the other series appeared in strain I sometime during the second generation after removal from the carcinogen. An exact record of its first recognized occurrence was not obtained. The degree of cell cohesion seen in strain I was in general comparable with that observed in strain H and was less than that observed in strain J. A detailed description, however, was not recorded.

Strain J.—Strain J was removed from strain D after 32 days in the carcinogen. The controls of this series were removed from the strain D controls at the same time. Even before these cultures were removed from strain D, the continuous width curve of the latter had shown a noticeable depression relative to its controls (fig. 6, sect. J). This depression continued uninterruptedly and at relatively constant level for 12 transfer generations following removal from the carcinogen. The average level during this time was 5.3 mm. Beginning about February 1943, however, the level of the curve became more erratic. The average level during this last period, through May 1943, was 7.4 mm.

The first record of a recognizable alteration in the cells of this strain was at 45 days after the first addition of carcinogen. At this time, of 10 cultures examined 4 showed definite cell cohesion; shortly thereafter all showed cohesion. Cohesion appeared earlier and was more severe than in strains H or I but was less severe than that in strains N or O, and continued to increase for possibly 60 days after removal of the cells from carcinogen, after which further change was less noticeable.

Strain K.—Strain K was removed from strain D after 55 days in carcinogen and after strain D had already shown a severe depression of the continuous width curve (fig. 6, sect. K). This depression continued at a relatively constant level as long as the strain was carried, that is, for five generations following removal from carcinogen. The average level of the curve from the second generation on was 6.6 mm. At 64 days after first

addition of the carcinogen, one flask of eight examined showed the cells of the culture to be typically coherent. This cohesion became increasingly severe, until at 139 days nearly all flasks showed typical cohesion and the formation of short strands, ribbons, and loose, fenestrated sheets. Later changes in the cells were progressive for a short interval after their removal from the carcinogen, but after this they showed no further recognizable progressive changes, nor did the cells show any recognizable loss of the changes already induced. The strain was closed at 237 days after the first addition of carcinogen. The controls of this strain appeared entirely normal as long as they were carried.

Strain L.—Strain L was removed from strain D, together with its controls, 111 days after the first addition of carcinogen. At this time its continuous width curve was showing the usual depression (fig.6, sect. L). The depression was much more severe than that for either strain H or strain I and was comparable with that shown by strains J and N. The curve continued at this low level through the ninth generation after removal from the carcinogen, the average level being 6.2 mm. During the next five generations (through May 1943) the curve showed erratic fluctuations, while the average level during this period was 9.5 mm.

The progression of cell changes observed in the cultures was similar to that described for the cells of strain N at comparable ages, up to the time of removal from the carcinogen, while after that time the cell changes were similar to but less severe than those of strain N.

Control Cultures

During the early part of the experiment, that is, until May 1942, the control cultures had been watched very closely and had appeared entirely normal. During the interval from May to July they were examined regularly in determining the width curves, but the cellular structure was watched somewhat less closely, more attention being given to increasing the number of cultures in the experimental series in order to study the behavior of the cells on reinjection into mice.

The first evidence of any abnormality in the morphology of the control cultures dated from about August 5, 1942, at which time three cultures in the strain H controls had definitely coherent architecture, typical of the cultures treated with methylcholanthrene. These three cultures were discarded. The other controls were normal at this time. On June 18 a series of normal cultures was reinjected into C3H mice, and some of them showed tumors after a latent period of about 60 days, that is, about August 18. It was only when the tumors appeared that a re-examination revealed a general slight cohesive alteration in the architecture of the control cultures. These alterations were of the same nature as those observed earlier in methylcholanthrene-treated cultures.

From August 18 to September 9, 1942, the normal continuous width curve for the control cultures remained at a relatively high level. About October 9, 1942, however, it showed a sharp depression which became increasingly severe until the middle of December and reached a level as low as that of strain H carcinogen-treated cells. The carcinogen-treated strains may also have shown some depression, but this was not conclusive. A critical visual examination of all control cultures was made in the latter part of August 1942. The findings may be summarized as follows:

(1) Of the various strains of control cultures, not one showed the normal loose structure that the controls had shown up to May 1942. In each strain a large fraction of the cultures showed recognizable but slight cell cohesion of the type which had been considered characteristic of the action of methylcholanthrene on these cells.

(2) The degree of alteration in some cultures was so slight that it could be considered only as probable rather than as clearly recognizable. In other instances there was no question but that the cells were cohering in the usual carcinogen-type architecture. In no instance was this

cohesion nearly so severe as that seen in the carcinogen-treated flasks of the H strain, which was the least altered of any of the experimental strains.

(3) No unaltered strain or line of cultures could be segregated from the rest of the controls.

(4) Not all strains were equally altered. The controls on strain H were probably the least altered of any, and they were so slightly altered that many of them seemed practically normal. In numerous instances, cultures which had arisen from the same explant at the last transfer showed distinctly different degrees of alteration.

Cultures From September 15, 1942, to May 1, 1943

A repeated detailed examination was made of all living cultures in late September and early October 1942, the cultures being studied at 2, 4, 11, and 16 days after planting. The conditions prevailing in cultures of the different strains are summarized herein, whereas the series of photographs are from a similar study made about December 17, 1942.

The descriptions of these various strains may be considered as characteristic. In all strains there was some overlap of the different cultures of different strains. For instance, in strain D an occasional culture was seen which was changed to a degree characteristic of strain H; strain H showed some cultures typical of those of strain D and some typical of strain J; similarly strain J had some characteristics of strain H and of strain L. Probably the closest overlap was with strains J and L, but even in these the average change for cultures of strain L was somewhat greater than that for strain J.

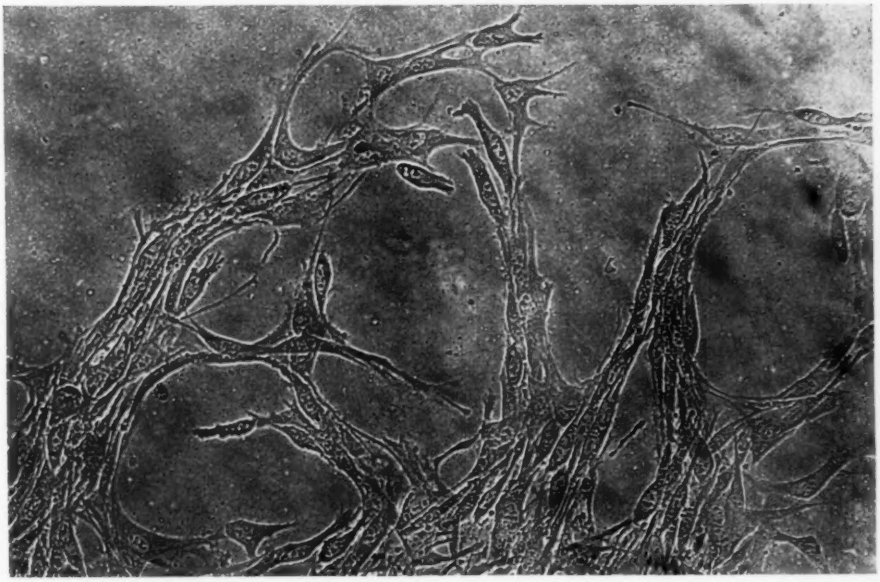

Figure 15.—Culture from strain D, photographed December 17, 1942, 9 days after planting. Note slight shortening of the cell processes and slight increase in lateral cohesion, with loss of the loose reticular structure of the normal fibroblast cultures and with the formation of loose cell strands. Compare with figures 9, *B*, and 11, *B*. × 200.

Strain D controls.—The architecture of the strain D controls was loose, although more coherent than that of the normal cultures at the beginning of this series of experiments in 1941. There were relatively few loose cells at the edges of the cultures, and there was a luxuriant growth at the glass interface. Growth at the fluid interface was irregular but in some instances was even greater than that at the glass interface. The characteristic growth at the latter is shown in figure 15. The cells were loosely coherent laterally and formed no definite ribbons but gave an architecture that lacked the characteristic reticular structure of the normal fibroblast cultures. The terminal processes of the cells were very slightly shortened and were amoeboid-appearing. There was usually no appreciable rippling or frothing of the lateral edges of the body of the cell, although the extreme tips and lateral edges of the terminal processes sometimes showed a slightly amoeboid pattern. The long axis of the cell was well-defined; only occasionally was a cell seen which departed from a more or less definite spindle or related shape; cytoplasmic granulation was not recognizably increased; nuclei were grossly normal; many mitoses were seen. Cell size seemed grossly normal although this estimate was extremely rough.

Strain H.—The growth of the cultures was luxuriant, practically indistinguishable from that of strain D controls; the structure was somewhat less loose (fig. 16), and the cells had a greater tendency to adhere laterally, forming more of a sheetlike structure in the culture. There were no loose cells at the edge of the culture, and the edge was sharp. The cells in general showed a spindle shape, although their terminal processes were shorter than those in strain D. The lateral edges of the terminal processes were often amoeboid along their whole length, down to where they joined with the body of the cell. The cytoplasmic granulation was not recognizably increased. Nuclei appeared grossly normal, many mitoses were seen, and the cell size seemed roughly normal.

Strain J.—There was a noticeable average reduction in the width of the growing zone of the cultures of strain J. The structure of the clump was altered from that of either of the preceding strains; the cell layer at the glass interface was often made up of characteristically slender interconnected loop

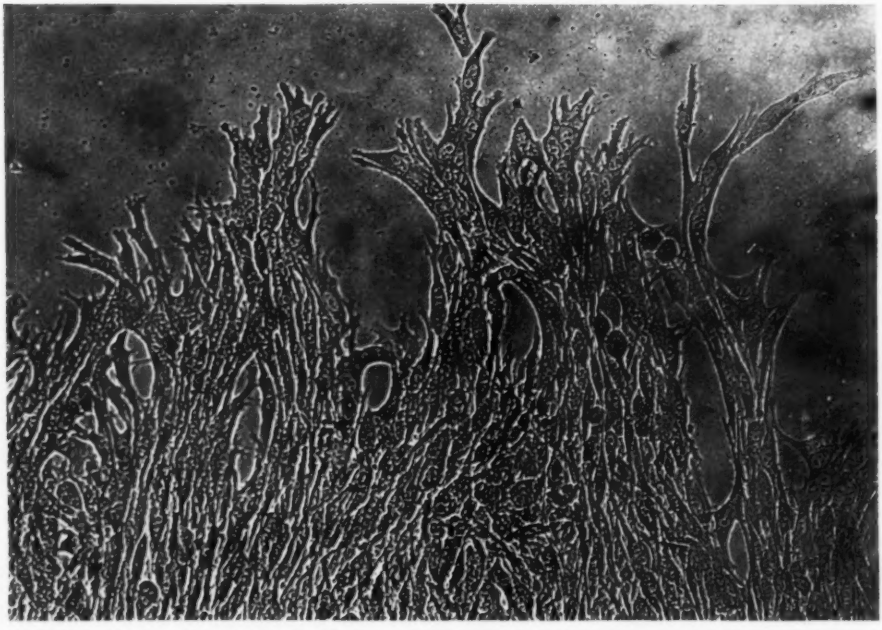

FIGURE 16.—Culture from strain H, 371 days after removal from the carcinogen and 9 days after planting. Note the progressive cohesion, shortening of cell processes, and amoeboid pattern of cell edges as compared with figures 11, *B*, and 15. × 200.

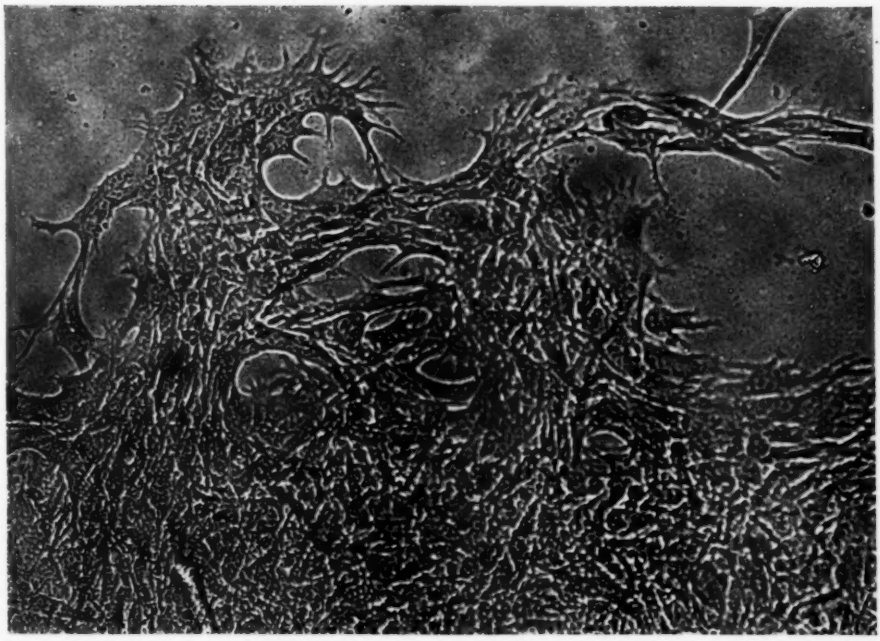

FIGURE 17.—Culture from strain J, 345 days after removal from the carcinogen and 9 days after planting. Note the progressive alteration from strain H (fig. 15). The clump of cells at the upper left illustrates the tendency of the cells at this stage to send off lateral processes, with the result that the cell axis is far less clear. × 200.

of cells. In other areas the cells were even more closely coherent laterally than in D or H. Such cells were no longer spindle-shaped but were more sheetlike, often with a number of lateral processes which gave them the appearance of having a far less definite axis (figs. 17 and 18). Cell processes were shorter, and their lateral and distal margins were frothy or irregular. They presented a pattern suggestive of amoeboid activity. The free edges of the body of the cell were also rippled or amoeboid in appearance. The growth at the edge of the culture was very much less clearly radial, the radial structure being distorted by the irregular loops and strands or ribbons of cells, which often were arched, interlaced, and fused, enclosing islands of clot. The granulation of the cells was only slightly greater than normal. Nuclei looked normal, and a few mitoses were observed, possibly fewer than in strains D and H.

Strain L.—In strain L the cells were even more sheetlike and more closely laterally coherent, tending to form sheeted ribbons and solid sheets, with fewer fenestrations and irregularities (fig. 19). At this stage they often resembled epithelial cells. The cell processes were even shorter than in the preceding stage, and the design of all free edges of the cell was frequently or usually suggestive of amoeboid rippling. Cell size, granulation, and nuclei did not seem greatly altered.

Strain N.—This strain was progressively altered from the preceding strains, and there was an even closer cohesion of the cells. Many cultures showed ribboning or combination of ribboning and sheet formation as the chief form of growth. There was a much greater tendency to sheet formation, and the sheets appeared thicker and denser (fig. 20). The edge was more regular than in strains J and L, and there was a tendency to form epitheliallike projecting lobes along the edges of the cultures. Cell processes were even shorter than in the preceding strain, but the amoeboid or frothy structure of the whole free edge of the cell was more accentuated, often so much so that the exact edge of the cell could not be clearly defined. There was a recognizable increase in the cytoplasmic granulation. Mitoses were fewer in number,

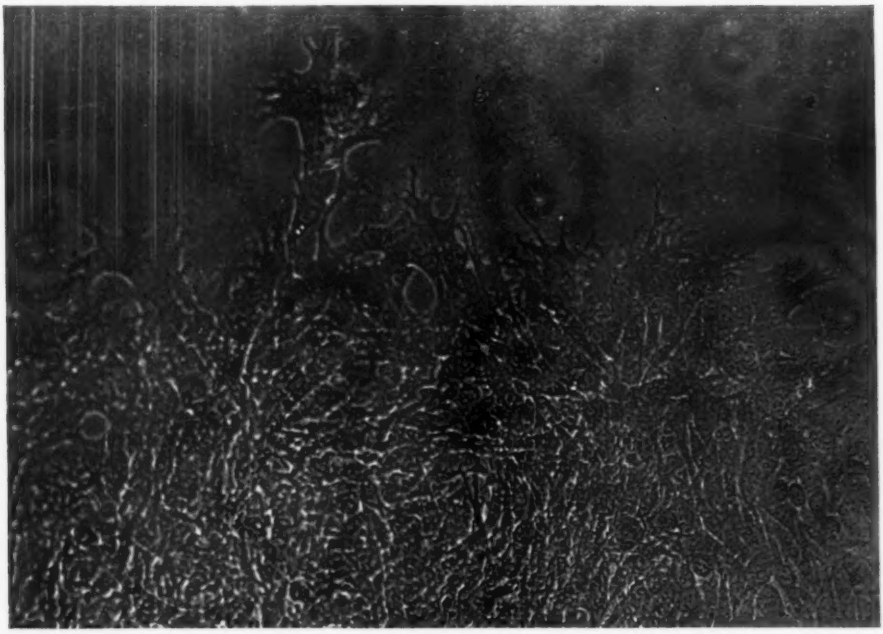

FIGURE 18.—Another culture from strain J, 345 days after removal from the carcinogen and 13 days after planting. This culture shows the tendency of the cells at this stage to send off lateral processes, with resultant obscuring of the cell axis. The photographic contrast is less as this was taken on a softer film. Approximately × 206.

and cells in mitosis were less rounded up and less separated from those around them.

Strain O.—The growing fringe of the culture in strain O was even narrower than in strain N, and the cells were more altered. Individual ribbons of cells or ribbons closely united into sheetlike structures were seen. The culture was often a mass of cells so closely united that it appeared almost as a syncytium (fig. 21), which terminated in an amoeboid-appearing edge which in some instances was lobose and epitheliallike, and in others broken only by very short amoeboid-appearing processes. (In stained preparations there was no evidence of a true syncytium.) Mitoses were often most difficult to recognize, as the cells showed little or no rounding up or separation from the surrounding cells of the sheet. Only in areas of less cell density could they be recognized. A careful examination of such areas revealed almost no cells in mitosis. In areas where the individual cells could be observed, nuclei were grossly normal. One, two, and three nucleoli were visible in each nucleus, and they appeared grossly normal. Nuclei were otherwise free from gross granulation. The cytoplasm was extremely granular, the granules being irregular in shape and size and easily visible with a 16-mm. lens. They often gave the cytoplasm a peculiarly moth-eaten and characteristic appearance. These granules were not to be confused with the occasional, small, highly refractile oil droplets seen in the cells, or with the slightly increased granularity seen in the early stages.

Examination of the cultures in December showed no recognizable progression of the later stages (L, N, O), but at that time there was thought to be possibly a slight progressive alteration of cultures in the earlier stages. For instance, numerous strain D cultures showed structure typical of strain H and in one instance of strain J, while strain H culture often showed structure typical of strain J.

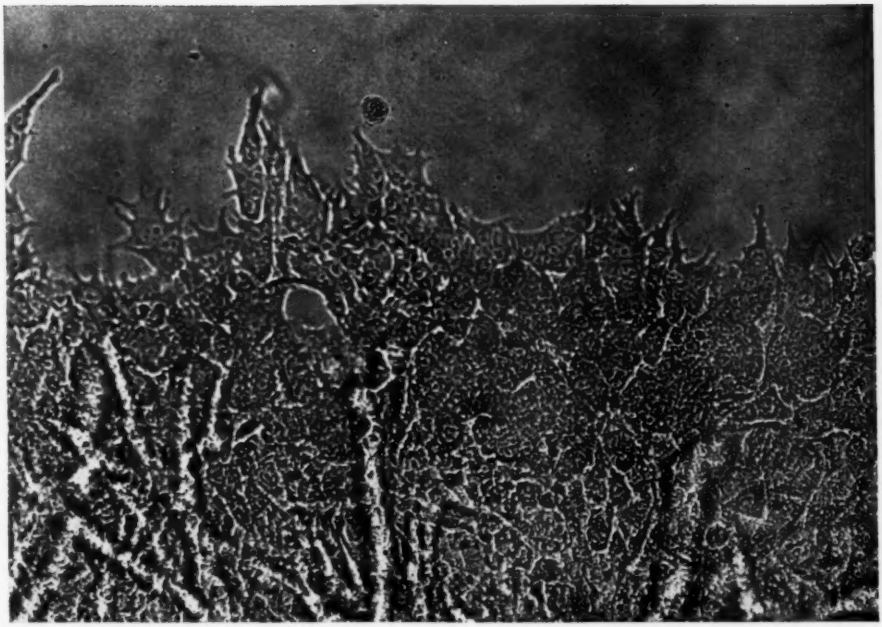

FIGURE 19.—Edge of culture from strain L, 266 days after removal from the carcinogen and 9 days after planting. Note progressive alteration from strain J, with formation of sheets and further shortening of cell processes. × 200.

Examination of the series May 15, 1943, showed the following results for the glass interface layers:

Strain D cultures were characterized by figure 15, a photograph of cells of strain D taken December 17, 1942. A few cultures were characterized by figure 16 (strain H).

Strain H cultures were characterized by figure 16, and a few by figure 15 (strain D control). None were observed showing the structure of figure 17 (strain J).

Strain J cultures showed a cell structure like those in figures 17 and 18 (strain J), and a few cultures were like figure 16 (strain H), while some showed coherent sheets of cells although the cell sheets were not so closely coherent as in figure 19 (strain L).

Most cultures of strain L presented a structure like that in figure 19 (strain L), and some like those in figures 17 and 18 (strain J). No cultures observed resembled figure 18 (strain N).

The degree of cohesion in strain N was definitely greater than in strain L. The cells were coherent, as in figure 20 (strain N), and often showed the same type of terminal edge. Cells were definitely more granular than the cells of strain L.

Strain O cultures were plainly more altered on an average than those of strain N. Culture edges similar to those in figures 20 and 21 were observed; the cells showed greater granulation than in strain N.

In all these cultures the fluid interface cells were looser and showed less coherence than did those at the glass interface. In strain N, two cultures were seen in which the retraction of the terminal and lateral cell processes had gone so far that the fluid layer cells had often lost all connection with each other and were separated and rounded up.

DISCUSSION

The cultures described by Earle and Voegtlin (*1*) were transferred at various

548963—43——5

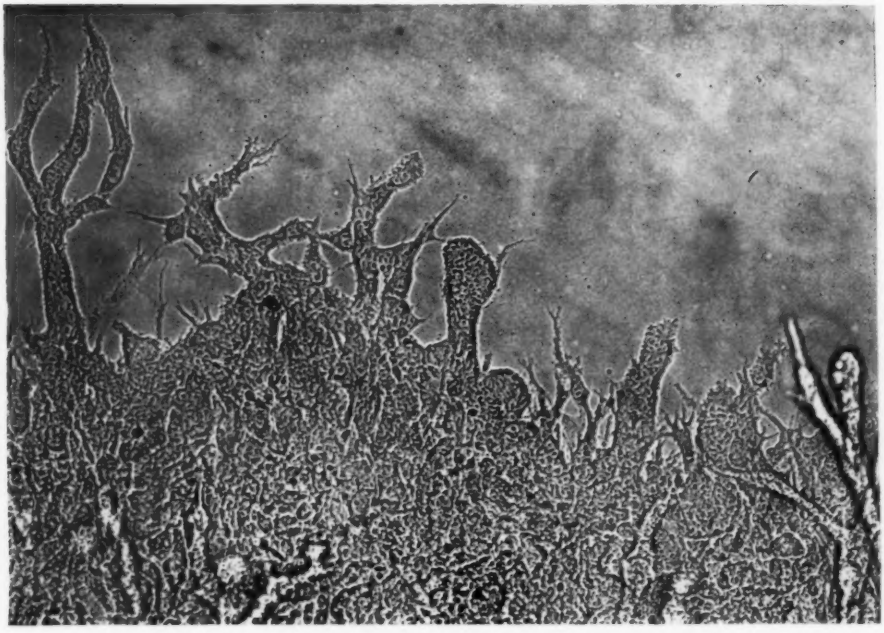

FIGURE 20.—Culture from strain N, 314 days after removal from the carcinogen and 9 days after planting. Note the extreme lateral frothing and rippling of the cell edges and the close cohesion of the cells into a sheet with definitely lobar edges. × 200.

intervals up to about 18 days. It was later felt that the frequency of transfer had interfered with the life of the cultures, and in the later series (*2*) length of the transfer interval was increased to as much as 112 days in some instances. This increase allowed the cultures to survive; but later work with these series and with other tumor strains indicated that the interval had been extended too long for the best life of the carcinogen-treated cells, while detail of the culture was obscured by the clouding of the fibrin clot. Accordingly, in the present studies the interval between transfers was reduced to 28 to 36 days. This period gave much better results than did either of the other two. About 30 days is probably the optimal common period for the strains of cells studied. Under the conditions used, even this period must be considered a compromise, since the normal fibroblast culture would probably give better results with transfer intervals of 36 days; while, because of central necrosis, carcinogen-treated cultures of very coherent architecture probably give better results with somewhat more frequent transfers.

In considering the results, the most outstanding question is that concerned with the behavior of the control cultures. In the earlier work (*1*, *2*), great care was taken in the handling of the dibenzanthracene and methylcholanthrene used. In spite of the precautions taken, just at the last of the experiments the control cultures showed a slight, though unquestionable, alteration in architecture. This alteration was similar to that observed in cultures

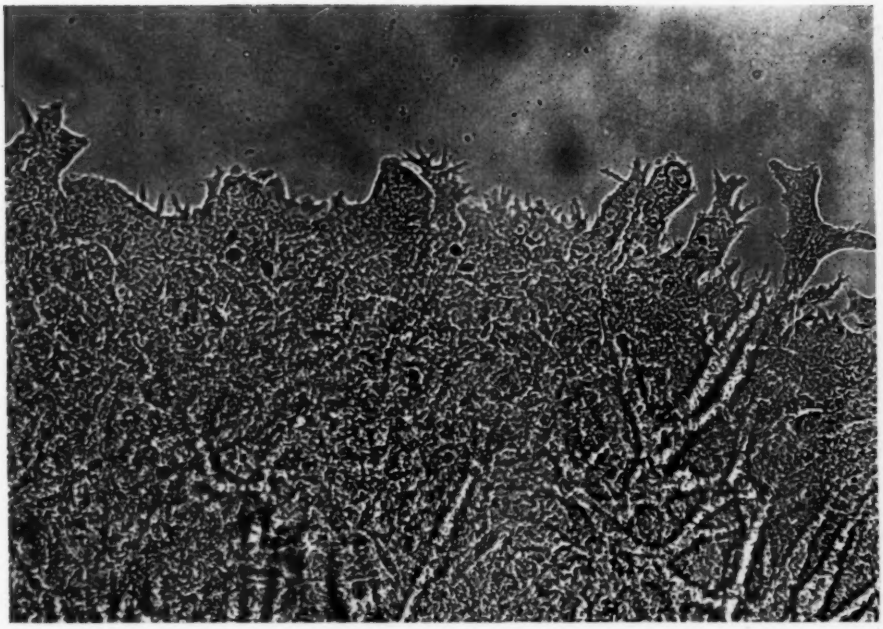

FIGURE 21.—Edge of culture from strain O, 92 days after removal from the carcinogen and 9 days after planting. Note the formation of a dense sheetlike edge often entirely devoid of cell processes for extensive lengths. × 200.

deliberately treated with methylcholanthrene, and the conclusion was reached that in spite of the care taken some trace of contamination of the controls with methylcholanthrene had occurred. In planning the present experiments an attempt was made to eliminate any possibility of contamination of the cultures with the carcinogen. The alteration of the control cultures must, therefore, be interpreted in the light of the care taken to avoid such contamination.

The possibility that the cells of the original parent strain used in this work were not normal cells of the fibroblast type but were malignant cells is most improbable because of the extreme rarity of subcutaneous tumors in this strain of mouse at the age at which it was used (100 days). It also seems improbable, since the appearance of the control cultures, certainly up to May 1942 (559 days after original planting), was entirely normal for fibroblasts from the subcutaneous tissue of mice and rats; and, when development of tumors in mice injected with control cultures led to careful examination of the cultures in August, there was no difficulty in recognizing that many of these control cultures had undergone a definite though slight change in morphology since their last detailed examination. The remainder of the control cultures showed suggestive but less conclusive morphologic changes, which in all instances became conclusive in those cultures that were carried on.

This alteration of the cells was of much less extent but was of the same morphologic type as that induced earlier in the strains of cultures treated with methylcholanthrene

and of the same type as the alterations we had formerly observed in tissues cultures of tumors arising in rats from the injection of methylcholanthrene.[2] It was also the same as that which Jacoby (*9*) had described for cells of tumors induced in vivo by the action of 1, 2, 5, 6-dibenzanthracene.

From these data it appears probable that the assumption of malignant properties by the control cells was closely associated with the alteration in the morphology of the cells which occurred about June 1942.

In accepting these original parent strain cultures as normal fibroblasts which underwent a morphologic and probably a malignant transformation about June 1942, several possibilities must be considered as to what induced this change. The possibility that the control cultures were contaminated with altered fibroblasts from the experimental cultures is eliminated by the unvarying order in which the cultures were handled, and by the fact that when the alteration in the controls was first observed the distribution of the altered cultures through the series was such that it could have been produced only by general contamination of all sets of the controls. This hazard seems entirely eliminated in view of the technical care employed. When the change in the controls was first observed, there was no sign of foci of greatly altered cells, distributed in relatively unaltered cultures. The appearance was rather that of a very slight, relatively uniform alteration of the cells, most easily recognizable at the glass interface of the culture. The possibility of contamination from any tumor cultures may similarly be dismissed since during the whole course of this experiment up to October 1942 no tumor cultures were being carried.

The possibility must be considered whether apparently normal fibroblasts of this strain of mice, when grown in a culture medium of chicken plasma, horse serum, and chick-embryo extract, can spontaneously go over into the altered form finally seen in the control cultures and give rise to sarcomas on injection.[3]

[2] *Unpublished work.*

[3] *Personal communication from Dr. G. O. Gey, outlining some results previously presented but never published.*

Gey, using a culture technique and culture medium of which the details are not at present available, describes alterations in several strains of cells, all of which arose originally from mesenchyme, of subcutaneous areolar tissue origin, from a young adult rat of pure strain Philadelphia albino stock. This stock of rat, according to Gey, rarely develops spontaneous tumors.

Three cell strains described showed spontaneous cell alterations; and when cultures of these altered cells were injected, they gave rise to tumors. In the first instance the alteration in the cells was first detected 4 months after primary explantation and "consisted of a change to cells varying greatly in size and with a great number of cells showing a typical and unequal mitosis. Inoculation of these greatly altered cells produced a variety of tumors different in cytological and cultural characteristics from their cells of origin and from each other. Transplantation from tissue culture to host and back again to tissue culture showed that each of the transplantable tumors was of a fixed type. Prolonged cultivation for long periods and further animal transfer revealed that they stayed fixed in type."

The third conversion occurred in a strain of cells related to the other two in that it came from the same small pieces of normal tissue. This strain had been under cultivation for several years without showing any changes of fixed type. When the change occurred, it showed up as microscopic areas of transformed cells within the thinly spread out periphery of only a few of the cultures. The transformation then spread through all the cells of the cultures during a period of several transfers and subcultures. There was thus produced a strain of malignant cells which on inoculation produced tumors of similar cell type. Other normal-appearing cultures of the same strain did not show any changes nor did they produce tumors on inoculation.

Gey also records that other normal cell strains of rat mesenchyme derived from another rat of the same stock were maintained for over 4 years without showing any change in type, and that to date he has not been able to reproduce these cell conversions by any deliberate experimental alteration of the cell culture environment. He states:

"The period of cultivation, whether months or years, is apparently unimportant. We have so far been unable to isolate any virus from these new malignant cell strains, Cell-free filtrates do not produce tumors on inoculations nor does the filtrate alter normal strains maintained in continuous culture. We were at one time inclined to believe that stray gamma radiation had some effect.

We know that the conditions in the tissue cultures used were different from those in vivo. The culture media and other culture conditions were such that the fibroblast continued to proliferate at a rate far greater than that in the adult animal. That this alone induced such a change in the cells seems unlikely in view of the extended work of Carrel and his associates (*10*, *11*, *7*), who carried fibroblasts from the embryo chick heart in cultures, in which they showed a rapid rate of growth for more than 25 years, apparently without any change in morphology. If these cells had undergone any such drastic morphologic changes as that seen in strains J, L, N, or O, they could hardly have been overlooked. It is emphasized, however, that whereas the Carrel strain of fibroblasts was from the heart of an embryo chick and was carried in solutions obtained from chicken blood and chicken eggs, in the present series of studies the cells were from subcutaneous tissue of a mouse and were carried during their whole period of culture in an entirely heterologous culture fluid. Obviously, in a number of respects the two sets of culture conditions were dissimilar, and the behavior of cells under the one might not hold for the other.

With the limited data available, no conclusion can be reached whether or not normal mouse subcutaneous fibroblasts by simple culturing in the heterologous horse serum-chick embryo extract can be induced to change over into sarcoma cells. This question is fundamental, and in answering it much important information concerning the mechanism of carcinogenesis may be obtained. Obviously, experiments designed to settle the issue should be carried out as soon as practicable.

The second possibility is that in spite of the precautions taken, some active amount of methylcholanthrene got into the control cultures and effected the transformation. The change noted in the control cells was more limited in degree but was of the same type as that induced through the action of methylcholanthrene. This fact, however, cannot be considered as evidence that the tumors arose from the action of methylcholanthrene, since Jacoby (*9*) noted a similar coherent architecture in cultures of malignant cells from a tumor induced in vivo by means of an entirely different carcinogen, 1, 2, 5, 6-dibenzanthracene, and the presumption is that a similar change could be effected with other carcinogens.

The evaluation of the possibility that the controls were contaminated is complicated by the fact that as yet no data are available on how little methylcholanthrene is necessary to change a strain of normal fibroblasts in tissue culture into one with such altered morphology and physiology. Bryan and Shimkin (*12*) have estimated that injection in vivo of about 2.4γ of methylcholanthrene is necessary to produce a sarcoma in strain C3H mice, in only about 1 percent of the injections, whereas 4.5γ produces tumors in about 5 percent of the injections. But conditions in vivo are different from those in vitro. It seems likely that a clump of fibroblasts in vitro is far more reactive or is under conditions far more apt to cause reaction with the carcinogen than are fibroblasts within the mouse. Hollaender, Cole, and Brackett (*13*) showed a definite photosensitizing ac-

Our observations to date on continuous weak irradiation do not confirm such a conclusion. Other factors are being investigated which may have some bearing on these unusual normal to tumor cell conversions which occurred in our cultures."

While Gey described a malignant alteration in several strains of mesenchymal cells, all from one rat, no conclusions are presented as to what induced such changes, nor do the data available indicate whether these transformations could have arisen from some trace contamination of the cultures with a recognized carcinogen. Attention is called to this work; but until more data are available, no attempt will be made to correlate it in detail with the results reported in the present paper.

tion of methylcholanthrene on yeast in the concentration of 10^{-9}. This reaction occurred within a few hours after addition of the carcinogen to the cells. In the absence of any data on the amount of methylcholanthrene necessary to alter a clump of mouse fibroblasts under the culture conditions in the present experiments, the possibility exists that when allowed to act on the cells for such an extended interval as a year or more, a concentration comparable with or even lower than that which these authors found active for yeast may be adequate. It is obvious that if such minute traces are active, the problem of treating strains of cells with the carcinogen for long intervals and of keeping other control strains entirely normal becomes most difficult.

While careful reconsideration of the technique used has given no definite reason to suspect any step in the handling of the cultures, the solutions, or the soiled glassware, less positive assurance is felt with reference to the re-use of rubber stoppers which were possibly contaminated with traces of methylcholanthrene. Although stoppers from carcinogen-treated cultures were never used on control cultures, the recleaning, sorting, wrapping, sterilizing, and other handling of these rubber stoppers may have introduced a trace-contamination hazard.

The laboratories in which this work was carried out are in the same building with other laboratories and animal rooms where large amounts of methylcholanthrene and similar carcinogens were handled. The design of the air conditioning and heating system of the building is such that there has been some trace recirculation of used air from many rooms, in some of which the carcinogen was certainly handled. Because of insufficient knowledge of the activity of low concentrations of methylcholanthrene and other carcinogens in tissue culture, the hazard of such trace contamination from this source also cannot be evaluated at present, although it is felt that its possible significance must not be overlooked.

The evaluation of another possibility, that the changes were induced by some unrecognized agent of unknown source, must await further work.

As the most probable explanation of the alterations in the control cultures and as a working basis for further study, the hypothesis is advanced that a trace contamination of methylcholanthrene occurred in spite of all precautions taken. If this is the correct explanation, the amount of carcinogen needed to effect such a change in the cultures is probably extremely small. It is suggested that, until more data are available, the most scrupulous care should be taken in all laboratories doing work with long-term tissue cultures so that such trace contamination of the cultures with methylcholanthrene or similar carcinogens does not lead to complications difficult to eliminate, and to erroneous conclusions.

With reference to the other observations reported in this paper, it seems clear that the changes in the control cultures arose at a substantially later date than the changes shown in cultures of even strains H and I, and more than 8 months after recognizable morphologic changes were observed in strains N and O. Furthermore, while of the same type, the changes in the controls have at all times been less accentuated than those seen even in strain H cultures. Regardless of whether the changes finally noted in the controls were spontaneous or arose from some contamination of the controls by the carcinogen, there seems no doubt that the cell changes observed in the experimental cultures are directly correlated with the action of the carcinogen that was delib-

erately added. This correlation is also shown by the orderly nature of the changes reported for different intentional exposures of the cultures to the carcinogen.

The retardation of growth and the degeneration of rat and mouse fibroblasts under the action of methylcholanthrene in various concentrations from (roughly) 0.2γ to 100γ have already been described by Earle and Voegtlin (*1*, *2*). These papers should be consulted for earlier references to the literature. Lebenson and Magat (*14*) carried out a further extensive series of studies on this point. They cultivated the skin of mouse embryos in chicken plasma and mouse and chick-embryo extract, in hanging-drop cultures, later shifting the skin to Carrel flasks. To these cultures was added 1, 2, 5, 6-dibenzanthracene, which was dissolved by letting the fluid plasma stand 2 days, with occasional shaking, in contact with an excess of the carcinogen. The cultures were left in contact with the carcinogen for intervals of 3 days and were then retransferred to normal medium and carried on. Some of the cultures were passed through dibenzanthracene once (from the fourth to the sixth day), whereas some received an additional exposure from the eighteenth to the twenty-first day. Cultures were apparently carried as long as 54 days.

During the first 3 weeks of growth no manifestation of the action of the carcinogen was noted. Most of the cells of the cultures were fibroblasts with a little epithelium. About the fourth week, culture growth improved considerably, and there was an intense liquefaction of the plasma in most of the cultures. Explantation of fragments of muscle onto the cultures gave rapid invasion of the muscle by the culture cells, destruction of the muscle, and a great acceleration of culture growth, the growth zone reaching 20 to 40 mm.^{2} in 3 days. There was also noted a greatly increased ability of very small clumps of cells to live and to grow rapidly.

Cultures were inoculated into mice from the twenty-second or the thirty-eighth to the fiftieth day after addition of the carcinogen. No mice showed tumors.

Lebenson and Magat concluded that while there was no clear demonstration of production of malignancy in their experiments, it was evident that the treated cells had acquired certain new properties under the influence of the carcinogen, and that the properties had appeared after a considerable latent period following treatment with the carcinogen. Furthermore, they considered that the induced changes were in a direction corresponding to an approach to the blastoma cell.

Larinov, Chertkova, and Samokhvalova (*15*), in an extensive series of studies, grew fibroblasts from the skeletal (femoral) muscles of new-born mice, in Carrel flasks, in a medium of chicken and rabbit plasma and dilute chick-embryo extract. To this medium benzpyrene or dibenzanthracene was added in the form of a fine suspension. Benzpyrene in a concentration of 10γ to 50γ per cubic centimeter gave a considerable inhibition of the growth of the fibroblasts, while dibenzanthracene displayed no such toxic action. For longer term tissue cultures, this carcinogen was used in a concentration of several gamma per cubic centimeter of culture fluid.

Cultures were carried up to 6½ months. There was nothing particularly distinctive in those exposed to the dibenzanthracene 17 days. When they were carried on without further addition of carcinogen, some of them began to grow far more rapidly at 40 days. Benzpyrene had no toxic action on them. In the following 3½ months the rapid growth continued, and

during this time many cultures were implanted in young mice. Of 55 mice inoculated, none developed a tumor.

In these treated cultures, frequently at the periphery of the culture zone, a culture would arise from a very small, isolated, clump of daughter cells. These daughter clumps grew very rapidly, and their cells showed a greater mobility, sometimes a larger size, and more disordered cell arrangement. In all cases the cells of these daughter cultures showed much more fat than did the parent cultures.

From our own experience with small cell clumps in culture, it is difficult to evaluate the significance of some of these findings of Larinov, Chertkova, and Samokhvalova. The increased storage of fat droplets within the cells is often seen in small clumps of normal fibroblasts (*16*), and in the present author's experience the cells of these clumps are often markedly less orderly in arrangement than are those in large clumps. The acceleration of growth of small clumps of cells treated with carcinogen agrees with the observations of Lebenson and Magat; and in the series of studies reported herein, the same phenomenon was frequently observed, at a stage of carcinogen treatment after definite cohesion of the cells had appeared. For instance, the cell clump shown in figure 12 continued to proliferate rapidly, while substantially larger clumps of control cells died.

The observations of Lebenson and Magat (*14*) and of Larinov, Chertkova, and Samokhvalova (*15*) and the observations reported in the present paper are in entire agreement that the cell transformations appeared after a definite latent interval of several weeks following initial exposure to the carcinogen.

Benevolenskaya (*17*) used cultures of embryonic chick heart and mesenchyme grown in hanging-drop cultures and in Carrel D3.5 flasks in a mixture of chicken plasma and chick-embryo extract, to which methylcholanthrene or 3, 4-benzpyrene was added, usually as a suspension. Concentrations of carcinogen ranged from 50γ to 1γ per cubic centimeter of culture medium, and cultures were carried in the carcinogen to 88 days. Frequently the cultures were transferred to normal culture media and carried for a time after this exposure to carcinogen.

Both carcinogens were toxic and induced stoppage of growth after 3 to 7 days in a concentration of 50γ per cubic centimeter. When treated cells were transplanted into a normal medium after such treatment, degeneration and death still ensued. As lower concentrations were tried the toxic action was less marked, until at 2γ per cubic centimeter the cultures were kept living in methylcholanthrene for 85 days, after which they were carried in normal media 65 days. Such concentrations proved slowly toxic. No changes in the cells were observed to suggest that they had undergone a malignant alteration; the changes were those of toxicity and degeneration.

Eight fowls were each injected with from 6 to 12 cultures that had been carried in a concentration of 2γ of methylcholanthrene per cubic centimeter from 37 to 62 days, and then carried without carcinogen 11 to 42 days, while 6 were each injected with 8 to 9 cultures that had been subjected to a concentration of 1γ of methylcholanthrene per cubic centimeter from 22 to 30 days and then carried 17 to 18 days in normal media. Nine chickens were injected with cultures that had been subjected to 2γ or 3γ of benzpyrene per cubic centimeter from 12 to 19 days, then carried from 6 to 37 days in normal culture medium, and injected. All injections were uniformly negative.

Cooper and Reller (*18*) treated the ears of mice twice weekly with a 0.6-percent

solution of methylcholanthrene in benzene. They found an increase in the frequency of mitosis of the epithelial cells from a normal average of 0.11 percent to 1.0 percent at 23 to 37 days after first treatment. This count subsided to 0.49 at 65 days and rose to 1.5 percent at 93 days, at which time the experiment was terminated. From 16 days on the ears became definitely hyperplastic and showed a fairly uniform thickening of the entire epidermis, and from the sixty-fifth day the ears of some of the mice began to show "precancerous hyperplasia." No definite carcinomas were observed within the duration of the experiment.

Creech (*19*), working with fibroblasts from connective tissue surrounding the rib of embryonic mice of pure strain, grew the cells for short periods as cover-glass preparations in a medium of fowl plasma and chick-embryo extract containing 20-methylcholanthrene-choleic acid or acenaphthene-choleic acid in concentrations of 100γ, 10γ, and 1γ per cubic centimeter. 1,2,5,6-Dibenzanthracene-choleic acid and phenanthrene-choleic acid were used in concentrations of 100γ and 10γ per cubic centimeter. The outgrowth of the cultures was measured at 45 and 70 hours, and relative areas of outgrowth were determined by camera lucida drawings and planimetric measurements. The cultures were finally fixed and stained and the cells studied. Nearly 1,700 cultures were studied. Methylcholanthrene-choleic acid in a concentration of 1γ per cubic centimeter (equivalent to 0.15γ of methylcholanthrene per cubic centimeter) and dibenzanthracene-choleic acid in a concentration of 10γ per cubic centimeter (equivalent to 1.5γ of dibenzanthracene per cubic centimeter) caused a significant increase in cell proliferation as indicated by measurements of outgrowth and by counts of mitoses, while the same carcinogens in a tenfold or greater concentration caused a retardation. Desoxycholic acid (10γ per cubic centimeter), phenanthrene-choleic acid (10γ and 100γ per cubic centimeter), and acenaphthene-choleic acid (1γ, 10γ, and 100γ per cubic centimeter) all caused a decrease in cell proliferation. With the methylcholanthrene and dibenzanthracene, a premature separation of the chromosomes in the prophase and metaphase was observed. This did not occur with the other substances tried.

The observation of Creech that methylcholanthrene-choleic acid retarded cell growth in a concentration equivalent to 1.5γ of the carcinogen per cubic centimeter accords closely with the data of Earle and Voegtlin for methylcholanthrene. The observation that a concentration equal to 0.15γ causes a significant increase in the rate of cell cleavage emphasizes further the necessity of investigating, over longer intervals, lower concentrations of the carcinogen than any yet used.

In the present work a concentration of 1γ of methylcholanthrene per cubic centimeter of fluid culture medium was used. With this concentration, after the first addition of the carcinogen to the cultures there was a sharp depression of the rate of increase in width of the cultures in every strain examined. The continuous width curves of the different strains showed that a great part of the depression of the curve was manifest by the end of the first 20 to 35 days after addition of the carcinogen. In strain N, which was continued in the carcinogen for as long as 184 days, the further depression of the continuous width curve was slight, while even in strain O there was no further depression recognizable until after 350 days in carcinogen, at which time there was a further sharp depression of the curve. This brought the rate of increase of width of the cul-

548963—43——6

tures to such low levels that further maintenance of the strain was most difficult. The strain would almost certainly have been lost during the next several generations if it had not been removed from the carcinogen.

Examination of the cells just after the onset of the initial sharp depression following the first addition of carcinogen showed no evidence of an increase in cohesiveness, nor was there any increase in cell density of the cultures. If there was any change in density, the cultures seemed somewhat less densely cellular than normally. Morphologic evidence of injury to the cells of young cultures, if it existed at all, was extremely slight. Certainly, there was no sign of widespread degeneration or disintegration of the cells. The depression of the curves, then, was not caused by any of these factors. While no determination has as yet been made of the actual rate of mitosis in the cultures, the data indicate a definite retardation in the frequency of mitosis in the cells. This would agree with Creech's data (*19*). Whether this retardation continued after cohesion of the cells set in, and up to 325 days after the first addition of carcinogen, was not determined and cannot be inferred from the data to date since the retarded rate of increase of the diameter of the culture was at least partially offset by an increase in cell density.

The data suggest, however, that such a retardation of mitosis did occur in cultures subjected to the carcinogen about 400 days (strain O) since the rate of increase of width of these cultures was greatly retarded, and few cells were observed in mitosis in the living cultures. The study of the action of the carcinogen on the rate of mitosis of the fibroblast for various intervals in long-term cultures under the conditions used must be left for future work. The conclusion is reached that, under the conditions of culture and of concentration of carcinogen used, the changes observed in the cells in the early stages (D, H, J, L) of action of the carcinogen are not recog-

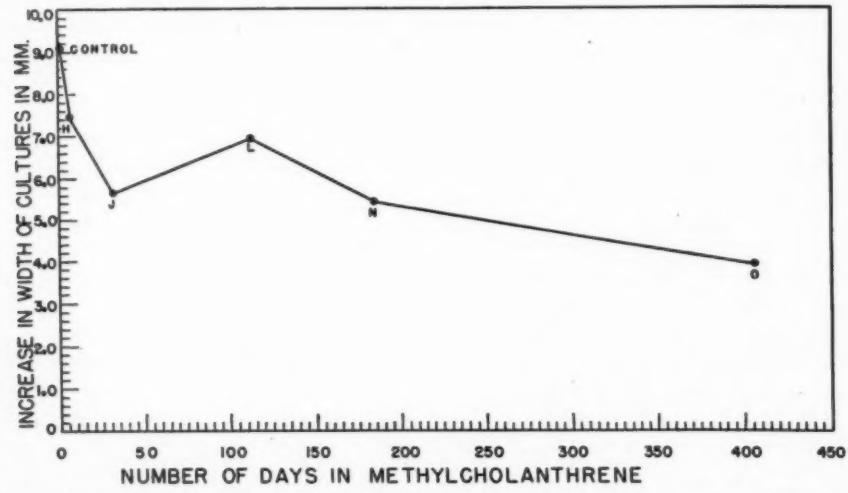

FIGURE 22.—Continued average level of the continuous width curves after removal of culture from carcinogen and through May 1943. The number of days the cultures were in the carcinogen is shown along the abscissa, the average culture width along the ordinate.

nizably such as to lead to the death of the cell. However, the cumulative, progressive action of the carcinogen for longer intervals and in the concentration used is not only clearly injurious but is almost certainly lethal.

In order to demonstrate more clearly the continued depression of the continuous width curves after removal of the different strains from carcinogen, the average level of the respective curves after removal of the strains from methylcholanthrene and through May 1943 is plotted against the interval of time each strain was subjected to the carcinogen (fig. 22). As a normal control point on these curves, the average of points on the continuous width curves, of the control cultures of strains D, H, I, J, K, L, N, and O through September 11, 1942, have been used. Values later than that have been discarded because of the depression in the curve after that date and the possibility that this depression resulted from trace contamination with the carcinogen.

The general trend of the curve is regular with the exceptions of point J (which is low) and point L (which is high). The longer the cells were left in the carcinogen, the less was their continued rate of increase of culture width after removal therefrom. The general morphologic appearance of the cells of strain J shows that they were more altered than those of strain H, but less altered than those of strain L, while the cells of strain L were less altered than those of strain N. This intermediate morphology confirms the correctness of the lateral position of points J and L along the curve. Other data (*3*) also indicate that this dip in the curve at point J and the rise at point L are real, although no explanation can be offered for this irregularity at the present time.

Leaving out of consideration the depression at point J and the rise at point L, the conclusion is obvious that, as far as is indicated by the continuous width curves, the degree of change induced in the cells with methylcholanthrene under the experimental conditions was a direct function of the duration of exposure (dose) to the carcinogen, and that even after the cell was removed therefrom, the induced changes persisted. In the instance of the cells of strains H, J, L, and N, this alteration has now persisted more than 1 year since their removal from carcinogen.

The question comes up whether or not there has been any rise at all in the continuous width curves since the cells were removed from carcinogen. The curve for strain H fluctuated over a wide range and showed no clearly defined rise. The curves of all the other strains maintained their low level for some time after removal from the carcinogen. This time included, for instance, 3 generations in strain O and 12 generations in strain J. From these times on, the curves lost their level, which had as a rule characterized them during the interval of carcinogen treatment and directly thereafter, and began to show erratic variations, and in some strains of cells, a rise. The significance of these variations is not yet clear, and the interpretation will be left to a later date.

From the various data presented, it seems evident that the cell changes previously described by Earle and Voegtlin have been confirmed, while the accumulation of additional data makes possible a clearer description of the sequence of the cell changes and of the later stages of the cells. As shown herein, in those strains left in the carcinogen for an extended period, at about 25 to 50 days after first addition of the carcinogen, there was a progressive shortening of the slender terminal processes of the cell, and an increased amoeboid appearance of the

lateral edges of these processes, an increase that extended farther and farther along the surface of the cell toward a zone midway of its length. Meanwhile the cells became more coherent, particularly laterally, and tended to form loose sheets and ribbons, then sheets, and finally massive, thick sheets, particularly prominent at the glass interface of the culture. The individual cells showed a progressively less clearly defined major axis, and there was a definite tendency (at about stage J, as shown in figures 17 and 18 (to send off amoeboid-appearing lateral processes. In later stages the cells were so closely coherent that the extremely short, amoeboid-appearing processes at the edge of the massive cell sheet were the only ones observed. In one or two instances in strain O, it seemed that the process of cell rounding had gone so far that the massive cell sheet or the heavy ribbons of cells had apparently disintegrated into living, isolated, rounded cells. Unfortunately, no photographs were made of this phenomenon. It is interesting to speculate whether such rounding and cell separation might increase the tendency of a tumor to metastasize in vivo.

This progression of changes, as described previously (*2*), was gradual and appeared to affect the whole culture rather than an isolated cell or a small clump of cells. There was no suggestion whatsoever of a sudden drastic change. The gradual change may be seen from a comparison of figures 15 to 21, inclusive. Furthermore, the changes, particularly those of cell cohesiveness and rippling of the surface, and changes in cell processes, seemed to be such as would arise from changes in the cell cytoplasm and more particularly in the cell surface.

The cells of strains H, J, L, and N, over 1 year after their removal from the 1γ concentration of methylcholanthrene, seem to have lost none of the typical cohesion and other morphologic changes induced with the carcinogen. While strain O has been carried for a shorter time since removal from the carcinogen, it, too, has preserved its structure. At eight generations after the removal from carcinogen, the cells of strain O were more altered than those of strain N, but the architecture of the cultures appeared possibly a trifle looser than it had directly after removal. This change was so slight, however, that at present it must be considered uncertain.

The conclusion is reached that over the extended period these cultures have been studied, once these morphologic changes have been induced in the cells by the action of the carcinogen, the cells to a very great degree seem to stabilize at that level or degree of alteration induced in their morphology.

While occasional instances of very large nuclei, or of very large cells, or of cells containing as many as eight very small nuclei were seen, usually these aberrations occurred in cultures more than 10 days old and were far more prevalent in those at about 25 to 30 days after planting. The impression was obtained from the living cultures that in the greater number of instances such aberrations occurred under culture conditions of cell overcrowding and degeneration. A more detailed study of nuclear changes will be made when the stained slides are studied.

Some carcinogen certainly persisted in the cultures for some time following their removal from it. However, in the more than 1 year that strains H, J, L, and N have grown since they were removed from methylcholanthrene at the concentration of 1.0γ per cubic centimeter, each has undergone more than 160 washings, more than 160 additions of fresh fluid, and 14 or more consecutive replantings. In each of the replantings it may be conservatively estimated that not in excess of one-fifth

of the original plasma (and cell) mass was transferred to the new culture. If any of the original carcinogen used on the culture still remains, it must be an extremely slight trace.

From the changes undergone by the control cultures, the presence of carcinogen in active though trace concentrations must be assumed as probable in the experimental cultures as well as the controls sometime in June 1942.

While a trace concentration of carcinogen might conceivably have had an effect on a normal cell, or on one which had had only a very low concentration of carcinogen for an extremely short interval, it appears most unlikely that such a trace contamination, if it occurred at all, has substantially altered the course of experimental cultures of strains H, J, L, N, and O. In spite of this, these strains and the altered controls have to a most remarkable degree stabilized at levels of alteration of the cells which are proportional to the time of their exposure to carcinogen. Minor changes which have occurred in the last few months or which may occur in the future find their most probable explanation in cell selection and in the overgrowth of more rapidly growing cell types within the cultures.

Bryan and Shimkin (*12*) injected methylcholanthrene in tricaprylin into the axillae of male mice of strain C3H and determined the final tumor incidence, as well as the time-frequency relationship, for various doses of the hydrocarbon. Table 1 is derived from the equations to their estimated relationships. The recognition of a tumor was determined when the cell mass reached the size of a palpable nodule which continued to increase in size.

Obviously these latent periods must each be considered as consisting of an initial transformative latent period during which the cells were changed from normal to malignant, and of a latent period of growth, an interval necessary for the cell mass to attain a palpable size. The data in another paper (*3*) show that the time required for subinoculated tumors, which were originally induced by the inoculation of cells treated with methylcholanthrene in vitro, to reach a palpable size is roughly 7 days. If this interval is accepted as being a rough index of the latent period of growth of such tumors and is subtracted from the latent periods given by Bryan and Shimkin, the time remaining may be considered a rough guide to the duration of the latent period of transformation.

The comparison of these results with those obtained from the treatment of cells in vitro with methylcholanthrene is obviously complicated by differences in concentration of the carcinogen and by the solvent used. In the results of Bryan and Shimkin (*12*), the minimal latent period for all doses of carcinogen ranges from 39 to 48 days, which would indicate a minimal transformative latent period of from 32 to 41 days. This period coincides with the period necessary to effect general characteristic morphologic alterations of the cells treated in vitro. The correlation, however, is incomplete since with Bryan and Shimkin's data the period from 32 to 41 days was the minimal, from

TABLE 1.—*Estimated dose-response relationships for 20-methylcholanthrene*

Mice with tumors (percent)	Estimated dose	Estimated latent periods		
		Extreme lower limit [1]	Extreme upper limit [2]	Average
	Gamma	*Days*	*Days*	*Days*
99	182	39	163	91
95	96	40	205	110
50	21	43	306	154
5	4.5	46	407	197
1	2.4	48	449	217

Derived from the data of Bryan and Shimkin (*12*).

[1] Time at which not more than 1 percent of the mice in the tumor population would be expected to have developed tumors.

[2] Time at which 99 percent of the mice in the tumor population would be expected to have developed tumors.

156 to 442 days the maximal, and from 84 to 210 days the average transformative latent period. The closer correlation of these results must await further work.

Grady and Stewart (*20*), working with pulmonary tumors induced with methylcholanthrene and dibenzanthracene in strain A mice, showed by section of the lungs that definite tumors appear about 5 weeks after injection. This latent interval for production of malignant tumors is in close correlation with the general period required to induce cell cohesion through the action of methylcholanthrene in vitro under the conditions of the present experiment.

Furthermore, confirmation that cell cohesiveness is intimately linked with the assumption of malignancy by the fibroblast is shown by some unpublished studies made on tumors that arose in vivo through injection of methylcholanthrene into rats. Several of these tumors were cultured for short intervals and showed definite cell cohesion. In a recent short study of a strain of tumor cells that originated from a subcutaneous injection of methylcholanthrene into a C3H mouse, cultures of the thirty-third passage of the tumors in mice showed a degree of cohesion roughly comparable with or slightly greater than that shown in strain D (control) cells (fig. 15).

Jacoby (*9*), using a pure strain of fibroblastic sarcoma cells derived from a spindle-cell sarcoma originally induced in mice with dibenzanthracene, described a similar cohesion of the cells. Jacoby's description, a part of which is quoted(*9, p. 301*), agrees with description of cell cohesion by Earle and Voegtlin and the changes in vitro reported herein.

> When grown in Carrel flasks in a hen plasma coagulum and fed with either Heparin hen plasma or a hen serum-chick embryo juice-Tyrode mixture, in which the embryo juice concentration is kept low, the cells regularly show a tendency to grow out in close association with one another, and to form ribbon-like strands or even broad sheets which resemble very much the epithelial type of growth *in vitro*. These ribbons frequently arborize and their branches often join up with one another, forming loops and bridges enclosing the coagulum, as shown in the accompanying illustration. The sheet-like growth is found especially at the interface coagulum-glass, whereas the cell ribbons occur also within the clot. This architecture seems to be very characteristic, and has been maintained through frequent passages. It permits the diagnosis of such a sarcomatous colony with the naked eye, being vastly different from the architecture of normal fibroblast colonies; aided by the more highly refractive cytoplasm of the tumour cells it brings the whole colony much more into relief against the background of the coagulum so that, even for the naked eye or under low power, the entire edge of the colony is sharply outlined and clearly defined.

The culture architecture shown in Jacoby's illustration is typically similar to some shown by Earle and Voegtlin (*2*) and comparable with a type of ribbon growth seen at about stage J or L of the present paper. It should be noted that at the time Jacoby's article was written, he had not seen the second one (*2*) by Earle and Voegtlin in which this cell cohesion was described.

These data all confirm the concept that the change in culture architecture is closely associated with the assumption of malignancy by the cells. This concept is further strengthened by the demonstration (*3*) that fibroblasts that have undergone a scarcely recognizable cohesion are capable of inducing typical sarcomas in a small percentage of injections into mice. The point cannot yet be considered as conclusively demonstrated, however, because, owing to the transformation of the controls, no study has as yet been possible to determine whether or not fibroblasts possessing the normal loose radial growth pattern can be made to produce sarcomas on injection.

Earle and Voegtlin (*2*) stated that the cohesiveness of cells under the action of

methylcholanthrene regularly occurred at both glass and fluid interfaces of the culture. Later data from all carcinogen-treated strains necessitated modification of this statement. Jacoby's recognition (*9*) that the cohesion was especially prominent on the glass interface of the culture is entirely in accord with later observations.

No definite relationship has been worked out for the relative growth of the cells at the glass and fluid interfaces, although the impression has been that in the carcinogen-treated culture up to about 12 days of age, the cells were particularly prominent at the glass interface, and often there was no fluid interface cell layer. The cells of the glass interface showed conclusively the increase in the cohesiveness of the cells from the action of the carcinogen. In some cultures, about 10 days old, the prominent layer of cells was at the fluid interface. When this occurred the cohesion was almost without exception less marked and particularly in cultures subjected to carcinogen for shorter intervals was sometimes unrecognizable. In cultures of an older age group the layer of cells at the glass interface of carcinogen-treated cultures was often overgrown or had become entirely necrotic; and in these cultures, too, the cells at the fluid interface were often not clearly cohesive, nor did they show so plainly such extreme shortening of the terminal cell processes in the early stages of carcinogenic alteration. They did show the granulation observed in the later stages. Because of these differences in the cells at the two interfaces, care must be taken to determine correctly the positions of the cells within the depth of the culture before reaching any conclusion as to the degree of carcinogen-induced morphologic alteration they have undergone.

The difference in the structure of glass and fluid layers is shown in figure 13, *A* and *B*, from the glass and fluid interfaces, respectively, of companion cultures of strain O at 403 days after addition of carcinogen and 11 days after planting. While these photomicrographs are entirely typical and illustrate the point, they are unfortunately not nearly so remarkable in contrast with each other as the two layers have often appeared, sometimes in the same culture in directly overlying regions.

The question arises why this increase in cohesiveness of the cells has not been commented on by other workers who have grown in vitro carcinogen-induced sarcomas which presumably arose from fibroblasts. The possibility is that this cohesiveness is prominent or occurs only in tumors that have arisen from certain strains of fibroblasts or from tumors that have arisen from a very limited group of carcinogens, or in a very limited group of culture media. The fact, however, that the cohesiveness is so much more prominent at the glass interface suggests another explanation which seems more likely. In tissue-culture preparations of the type used (in Carrel flasks) the thickness of the fibrin clot is substantially greater than the thickness of the clot in either "roller tube" cultures (*21*) or in many, if not all, hanging- or lying-drop slide preparations. This would give a greater separation of glass and fluid interface cell layers. Since the glass interface layer is the one closer to the microscope objective, in the flask culture it naturally assumes a prominence, definition, and isolation which are probably not so accentuated in other types of cultures. With many slide cultures the extremely short interval during which the cells may be carried undisturbed, usually just a few days, would itself interfere greatly with the appearance of this cohesion architecture as it is most easily recognized at about 8 to 10 days of culture.

A further question is why this change in cell cohesiveness should be more promi-

nent at the glass than at the fluid interface. While with the data available differences in such factors as oxygen tension or relative nutrition at the two levels in the plasma clot cannot be ruled out, it seems more probable that the difference lies in the reaction of the surface of the cell to interfacial forces, which would be different at the plasma-fluid interface from those at the plasma-glass interface.

Central necrosis in carcinogen-treated cultures appeared regularly at an earlier date than in the controls. It usually appeared first at the glass interface. This central necrosis seemed to result from the extremely compact architecture of the culture with consequent crowding and interference with the metabolism of the deepest and most central cells. Young cultures, where the cells were not overcrowded, appeared to be free of necrosis.

During the whole course of the experiment, there was no detectable increase in the rate of liquefaction of the clot, nor did any cell strain show an increase. A similar absence of unusual liquefaction has also been noted in other malignant cells grown in this chicken plasma-horse serum- chick embryo extract medium.

In considering the nature of the cell changes observed, it is desired to call attention to certain points. These are: (1) The drastic morphologic changes in cells treated with carcinogen resulted in the production of cell types greatly altered from the original cell type used; (2) the morphologic changes were gradual; (3) they could be considered as changes that might arise from alteration in the cell surface; (4) these changes stabilized after the removal of the cells from carcinogen, and different cell strains treated for different periods stabilized at different levels of morphologic alteration; (5) the morphologic changes were correlated with the action of a definite, highly purified, crystalline chemical substance in extremely low concentration; and (6) the changes were induced in cells in an entirely heterologous culture medium quite removed from the systemic influence of the parent host.

With our present lack of knowledge these considerations cannot lead to any definite conclusion. Even with the recognition of this fact, the suggestion is made that the cell changes observed may well be of the same category as those seen in the process of cell differentiation of the organism, and it is emphasized that these changes are considered by some workers to result from definite chemical organizers acting in extremely low concentrations. It is felt that this similarity is too close to be overlooked or to be dismissed without most careful consideration and that the data call for further evidence either to confirm the similarity or to reveal dissimilarities.

SUMMARY

A primary strain of fibroblasts from the subcutaneous and adipose tissue of a 100-day-old male mouse of the C3H strain, Andervont substrain, was started in vitro in horse serum-chick embryo extract-saline solution on October 18, 1940. Cultures were grown in Carrel D3.4 flasks, slightly modified. When the strain reached an age of 291 days in vitro, the first addition of purified 20-methylcholanthrene was made to selected groups of the cultures in a solution of such strength that the final concentration of carcinogen in the fluid culture medium was 1γ per cubic centimeter.

Various selected groups of cultures were carried in the carcinogen for different intervals. Particular attention is directed to cell strains designated H, J, L, N, and O, which were subjected to the carcinogen for 6, 32, 111, 184, and 406 days, respectively, at the end of which time they were carried on without further addition of

carcinogen. To June 1, 1943, these strains had been carried 542, 542, 542, 663, and 663 days, respectively, since first addition of carcinogen, and 536, 510, 431, 479, and 257 days since their removal from it.

The first noticeable effect of the carcinogen was a definite decline in the rate of increase in width of the cultures. This initial reaction appeared after a very few days in the carcinogen and in its preliminary stages at least seemed to arise from a retardation of cell proliferation. This depression of the rate of increase of size of the cell clump continued even after the cells were removed from methylcholanthrene. It appeared that the depresssion of the rate of cell proliferation was also present at about 400 days in the carcinogen, but whether the depression of the rate of increase in width of the cell clump arose from this factor alone was not determined. The conclusion is that the action of the carcinogen on the cells was progressive and gradual, and in the course of time almost certainly lethal.

The earliest recognizable morphologic change in the cultures appeared about 40 days after first exposure to the carcinogen. In general, the cells showed a gradual diminution in the length of the terminal cell processes. The lateral edges of the processes became increasingly amoeboid in appearance, and this change extended progressively from the tips of the cell processes toward the middle part of the cell as time in the carcinogen went on. The cells gradually tended to send out numerous short lateral processes at their free edges; meanwhile the cells became increasingly coherent, particularly laterally, with the production of cell strands, cell ribbons, and finally of sheets which showed many characteristics of epithelial sheets in tissue culture. As time went on, the sheets became increasingly massive and the cell cytoplasm extremely granular.

The changes induced in different cell strains with different intervals of exposure continued after the carcinogen was discontinued. To date, four cell strains, subjected to the carcinogen in the concentration mentioned for 6, 32, 111, and 184 days, respectively, have been carried for more than a year since the carcinogen was discontinued, without loss of their induced characteristics. One strain has not been carried so long without carcinogen, but it also shows no clearly defined diminution.

Over 8 months after the first recognizable morphologic cell alterations in the earliest group of experimental cultures (strain N) and approximately 2 months after the last experimental strain (H) had shown recognizable cell alterations, the control cultures started to show definite alteration of the same type as but to a lesser degree than the experimental cultures. Of the various control strains examined, strain H control showed least alteration, the change in the architecture of the cultures of this strain being barely discernible morphologically. The possibilities whether this change arose from a spontaneous tendency of the apparently normal fibroblast to undergo a malignant change under cultivation in vitro or whether the change was induced from trace contamination of the control cultures with carcinogen, are discussed. The data available do not permit a definite conclusion, but the working hypothesis is advanced that such trace contamination with carcinogen did occur, although the mechanism by which it occurred is unknown. In view of the precautions taken to guard against contamination, it is emphasized that the contamination, if it occurred, was probably extremely slight. Until further data are available to define more clearly the active concentration of carcinogen necessary to induce changes in cells in long-term tissue cultures, great care should be taken in the

handling of such cultures with carcinogen to eliminate the possibility of complications that might arise from trace contamination.

After all deliberate treatment of the cells with carcinogen had been discontinued, and after strains H, J, L, and N had been carried for more than a year after discontinuance of the carcinogen whereas the other strains had been carried for shorter periods, the characteristics of the control cultures and cultures subjected to carcinogen for 6, 32, 111, 184, and 406 days were compared. The progression of morphologic changes with longer exposure to the carcinogen was similar to that described previously.

The similarity of the latent period for the earliest in vivo production of tumors with methylcholanthrene and the time necessary for the production of recognizable morphologic alteration of the cells in vitro is pointed out.

REFERENCES

(*1*) EARLE, W. R., and VOEGTLIN, C.: The mode of action of methylcholanthrene on cultures of normal tissues. Am. J. Cancer, 34: 373–390 (1938).

(*2*) EARLE, W. R., and VOEGTLIN, C.: A further study of the mode of action of methylcholanthrene on normal tissue cultures. Pub. Health Rep., 55: 303–322 (1940).

(*3*) EARLE, W. R., and NETTLESHIP, A.: Production of malignancy in vitro. V. Results of injections of cultures into mice. J. Nat. Cancer Inst., 4: 213–227 (1943).

(*3a*) NETTLESHIP, A., and EARLE, W. R.: Production of malignancy in vitro. VI. Pathology of tumors produced. J. Nat. Cancer Inst., 4: 229–248 (1943).

(*4*) EARLE, W. R.: Production of malignancy in vitro. I. Method of cleaning glassware. J. Nat. Cancer Inst., 4: 131–133 (1943).

(*5*) ———: Production of malignancy in vitro. II. Photomicrographic equipment. J. Nat. Cancer Inst., 4: 135–145 (1943).

(*6*) EARLE, W. R., and CRISP, L. R.: Production of malignancy in vitro. III. Microcinematographic equipment. J. Nat. Cancer Inst., 4: 147–164 (1943).

(*7*) PARKER, R. C.: Methods of tissue culture. Paul B. Hoeber, Inc., New York (1936).

(*8*) CUNNINGHAM, B., and KIRK, P. L.: Measure of "growth" in tissue culture. J. Cell. & Comp. Physiol., 20: 343–358 (1942).

(*9*) JACOBY, F.: Architecture of colonies of a pure strain of fibroblastic sarcomatous cells derived from a dibenzanthracene mouse tumor. (Letter to the editor.) Nature, London, 146: 301–302 (1940).

(10) EBELING, A. H.: A strain of connective tissue seven years old. J. Exper. Med., 30: 531–537 (1919).

(*11*) ———: A ten year old strain of fibroblasts. J. Exper. Med., 35: 755–759 (1922).

(*12*) BRYAN, W. R., and SHIMKIN, M. B.: Quantitative analysis of dose-response data obtained with three carninogenic hydrocarbons in strain C3H male mice. J. Nat. Cancer Inst., 3: 503–531 (1943).

(*13*) HOLLAENDER, A., COLE, P. A., and BRACKETT F. S.: Absorption and fluorescence spectra in relation to the photolethal action of methylcholanthrene on yeast. Am. J. Cancer, 37: 265–272 (1939).

(*14*) LEBENSON, E., and MAGAT, M.: Changes in the biological properties of cells in skin cultures of mouse embryos upon prolonged action of dibenzanthracene. J. Méd. de l'Acad. d. sc., RSS d' Ukraine, 7: 381–391 (1937). [In Russian, English summary, pp. 389–391.]

(*15*) LARINOV, T., CHERTKOVA, M. A., and SAMOKHVALOVA: Alterations of the biological properties of cells in tissue cultures under the action of cancerogenous substances. Bull. biol. et med. exper. de l'U. S. S. R., 9: 515–517 (1940).

(*16*) EARLE, W. R., and THOMPSON, J. W.: The influence of the size of the explant upon cultures of chick fibroblasts in vitro. Pub. Health Rep., 45: 2672–2698 (1930).

(*17*) BENEVOLENSKAYA, S. V.: The effect of methylcholanthrene and 3:4-benzpyrene on cultured chick mesenchyme. Arch. d. sc. biol., 57: 94–105 (1940). [English summary, p. 105.]

(*18*) COOPER, Z. K., and RELLER, H. C.: Mitotic frequency in methylcholanthrene epidermal carcinogenesis in mice. J. Nat. Cancer Inst., 2: 335–344 (1942).

(*19*) CREECH, E. M. H.: Carcinogenic and related non-carcinogenic hydrocarbons in tissue culture. Am. J. Cancer, 39: 149–160 (1940).

(*20*) GRADY, H. G., and STEWART, H. L.: Histogenesis of induced pulmonary tumors in strain A mice. Am. J. Path., 16: 417–432 (1940).

(*21*) GEY, G. O., and GEY, M. K.: The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: cultivation of mesoblastic tumors and normal tissue and notes on method of cultivation. Am. J. Cancer, 27: 45–76 (1936).

Production of Malignancy in Vitro. V. Results of Injections of Cultures into Mice

By WILTON R. EARLE, *senior cytologist*, and ANDERSON NETTLESHIP, *passed assistant surgeon* (*R*), with the technical assistance of EDWARD L. SCHILLING, THOMAS H. STARK, NANCY R. STRAUS, MARY F. BROWN, and EMMA SHELTON, *National Cancer Institute, National Institute of Health, United States Public Health Service*

In the preceding paper (*1*) Earle described the growth of cultures of a primary strain of fibroblasts, from the subcutaneous and fatty tissues of a young strain C3H male mouse, in a completely heterologous medium. Different groups of the cultures were treated with a concentration of 1.0γ of 20-methylcholanthrene per cubic centimeter of culture fluid for different periods. As a result of these studies, a number of strains of cells were originated: Strain H, treated with carcinogen 6 days and removed therefrom December 11, 1941; strain J, treated 32 days and removed January 6, 1942; strain L treated 111 days and removed March 26, 1942; strain N, treated 184 days and removed February 6, 1942; and strain O, treated 406 days and removed from carcinogen September 16, 1942.

The control groups of strains never received any known treatment with the carcinogen, but about June 1942, after cultivation in vitro approximately 20 months, the control strains showed changes which suggested that a trace contamination with the carcinogen had occurred. Strain H controls (designated strain HW) were so slightly altered morphologically that the change was barely recognizable; strains D, J, L, N, and O controls were altered morphologically to a recognizably greater degree than the strain H controls, but substantially less than the strain H experimental cultures.

For the techniques used in handling these strains and the description of the strains in vitro, reference is made to the preceding paper (*1*). The present paper summarizes the data obtained on reinjecting cultures of these strains into young strain C3H mice, 2 to 6 months old.

MATERIALS AND METHODS

All inoculations were made from cultures that had reached the normal time for transfer to fresh flasks, that is, from cultures 28 to 35 days old. Each culture was removed from its flask in the manner previously described (*1*), the heavy ridge at the edge of the fibrin sheet was trimmed off, and areas of the clot which obviously contained no cells were also removed. In the early experiments, the remaining cellular area was cut into strips comparable in size with those used in the transfer to fresh flasks, about 4 x 20 mm., and these strips were placed in about 0.7 cc. of saline solution in a depression plate. Later, the cell sheet was left intact. At the end of the dissection of a set, each explant was loaded into a 3½-inch, 13-gage hypodermic needle, which was fitted with a heavy plunger. These needles were used as trocars to inject the cell mass.

Since there was some possibility that the cells would dry out in these trocars and since much trouble was experienced in loading, after the first few experiments 1-cc. tuberculin syringes fitted with 18-gage needles 1½ inches long were used instead of trocars. Several methods of

loading the syringes were tried: At first the cell clump was deliberately broken up by sucking it up through the needle into the syringe, but this procedure was often troublesome; later the clump was lifted out of the saline with a platinum-iridium spatula and slipped into the rear end of the barrel of the tuberculin syringe through a small funnel (drawn down from glass tubing of 12-mm. diameter) about 50 mm. long and sloped gradually so that it measured 4 mm. inside diameter at its tip. This latter procedure has proved satisfactory. Whatever the technique used in preparation and injection, each culture was handled separately, with separate dissecting dishes, knives, trocars, syringes, and funnels, so that there was no cross contamination from one culture to another.

All mice were of the C3H strain, Andervont substrain, and were obtained from Dr. H. B. Andervont or Dr. J. W. Thompson, of this Institute. All were fed Purina dog chow. Parallel injections of the mice from the two sources showed no difference in their behavior so the source will not be specified for each lot. The mice in the experiment were individually labeled and were kept in a room where no other tumor-bearing mice were placed.

For each mouse, the area of skin through which injection was to be made was clipped and wiped first with absolute alcohol and then with 1:500 mercuric chloride, or in later work with a 1:500 solution of metaphen. This solution was wiped off with alcohol and injection made through the still damp area. The site was then swabbed with a thin layer of petroleum jelly to prevent injury to the skin from the alcohol.

The injected mice were examined about once a week although there was some variation in this, examination being less frequent and regular in the early experiments than in the later. The infrequency of examination must be taken into consideration in interpreting the time recorded for a tumor to appear after inoculation. All tumor sizes were determined with calipers graduated to millimeters, and the three axes of the tumor were measured as accurately as practicable.

With the exception of mice which were lost or which died overnight or over a week end and were too badly decomposed to permit autopsy, all were killed and autopsied. In early experiments mice were frequently killed early after tumor appearance. In later experiments the mouse was usually killed either when tumor size indicated that the animal could not live long or when the mouse had been carried at least 90 days after injection.

Autopsies were carried out with special attention to the area around the site of injection and with respect to the possibility of metastases. The pathology is given here only to the degree necessary to establish diagnosis, as it will be reported more fully in the next paper (*2*). When a diagnosis of sarcoma is given, it is meant to indicate a structure of the tumor characteristic of sarcoma as determined by microscopic examination, with definite invasion of the surrounding tissue. When the tumor is described as regressing, the term is used to indicate such general cell injury, degeneration, and necrosis, as to suggest that the tumor would possibly have been resorbed.

In all subinoculations of tumor tissue from mouse to mouse, the trocars described have been used. Nearly all subinoculated mice were also autopsied.

RESULTS

Primary Inoculation

In the early experiments on the injection of these strains of cells, the primary aim was to try to demonstrate the production

of tumors from the cells treated with carcinogen in vitro. Since the potentialities and behavior of these cell strains in vivo were entirely unknown in the early experiments a number of different variations were introduced in the methods of injection and handling of mice to find what seemed to be optimal conditions for tumor production. In view of the earlier work of Earle and Voegtlin (*3*) with the Jensen rat sarcoma, the large majority of the injections were intramuscular.

Experiment 1

Cultures of strain N carcinogen-treated cells were injected (March 3, 1942) 25 days after the carcinogen was discontinued. Injections were made by trocar into the back muscle; one-half culture (two culture strips) for each male mouse was used. The cultures had been grown during the preceding generation in contact with normal control cells of the same strain in the horse serum-chick embryo extract medium already described (*1*). The whole cell mass including normal and strain N experimental cells was injected.

Nine days after injection, six of the eight mice injected showed small tumors at the site of injection. At autopsy, 121 days after injection, five of the eight mice showed tumors, ranging in size from one that measured 1 x 1 x 1 mm. along the three axes up to three much larger tumors, of which the largest measured 20 x 15 x 15 mm. All five tumors were diagnosed microscopically as sarcoma; one (5 x 5 x 2 mm.) appeared to be regressing. The remaining three mice were normal.

Experiment 2

Cultures of strain N carcinogen-treated cells were injected (May 26, 1942) 109 days after the carcinogen was discontinued. Injections were made by trocar into the back muscle and subcutaneous regions of male mice. This experiment included three groups of mice as follows:

Group 1.—Cultures were injected into 29 normal mice. Four showed tumors about 5 x 4 x 3 mm. in size 9 to 14 days after injection. On autopsy at the time of tumor appearance, all were diagnosed sarcomas; 3 appeared microscopically to be regressing. Three other mice showed tumors at 24, 44, and 86 days, respectively; the largest tumor measured 17 x 17 x 12 mm. at autopsy shortly after it was first observed (86 days). All 3 were diagnosed sarcoma; 1 appeared to be regressing. One mouse was negative when autopsied at 45 days; and 21 were negative when autopsied at 86 days.

Group 2.—Cultures were injected into 16 mice which 24 hours earlier had been subjected to 400 r of hard X-ray [1] (200-kv. 0.5-mm. copper filter) applied to the whole body. This was done in the hope of facilitating growth of the tumors by lowering the resistance of the mice.

Nine mice of this group showed tumors at 9 to 14 days. One mouse died and was not examined; eight were autopsied at from 9 to 44 days after injection, and the smallest tumor was 3 x 2 x 2 and the largest 20 x 15 x 9 mm. Two additional mice showed tumors at 21 and 86 days, which reached a size of 15 x 8 x 6 mm. and 10 x 7 x 3 mm. at autopsy at 44 and 86 days, respectively.

Group 3.—Cultures were injected into 19 mice which 24 hours earlier had received an intraperitoneal injection of 1 cc. of 60 percent horse serum and 40 percent embryo extract. This group was inoculated to explore the possibility that by desensitizing the mice, to some extent, to the medium used in growing the cells, the growth of the tumors could be facilitated.

Two mice showed small tumors in 9 days and 2 in 86 days. All were autopsied at the time of the first recorded appearance of tumor. Microscopic examination of 3 of these 4 mice led to diagnosis of sarcoma, while the fourth was not sectioned. The other 15 mice were negative when autopsied at 86 days.

A great deal of trouble was experienced in this and other early experiments with irradiated mice. Irradiation apparently so greatly lowered the resistance of the mice that an epidemic of something like mouse typhoid started in the irradiated group and spread to the other two groups of this

[1] *These and other irradiated mice used were irradiated by Dr. Paul S. Henshaw, of this Institute.*

series. All mice which seemed at all unhealthy were, therefore, killed. Such mice are omitted from the summarized data. Because of this complication, the use of X-ray irradiation on groups other than those on which it had already been started, was discontinued. The use of a previous injection of horse serum and chick-embryo extract was also discontinued in further work as it seemed to offer no advantages.

Experiment 3

Cultures from strain L carcinogen-treated cells were injected June 2, 1942, 68 days after the carcinogen was discontinued. Injections were made by syringe into the back muscles of male mice. This experiment included two groups:

Group 1.—One-half culture was injected into each of 19 mice. In 22 days 1 mouse showed a tumor, which reached a size of 20 x 15 x 6 mm. at 43 days. Autopsy at this time gave a diagnosis of sarcoma. One mouse was negative when killed at 37 days. Seventeen mice carried to 79 days were negative at autopsy.

Group 2.—One culture was injected into each of 14 mice. Six showed tumors in from 14 to 37 days, and three others at 79 days. Three of these tumor-bearing mice died without autopsy. Six autopsied at 22 to 79 days showed tumors ranging from 15 x 7 x 6 to 28 x 25 x 14 mm. in size. Six tumors sectioned all gave a diagnosis of sarcoma. Five mice were negative when autopsied at 79 days.

Experiment 4

Cultures used in experiment 4 were of control cells from strains O and K, which were injected (June 18, 1942) by syringe into the back muscles of male mice. This experiment included four groups.

Group 1.—One-half culture was injected into each of three normal mice. All were negative when autopsied at 149 days.

Group 2.—One whole culture was injected into each of five normal mice, one of which showed a tumor in 14 days. The diagnosis at autopsy at this time was regressing sarcoma. Three mice showed tumors in from 50 to 64 days, with maximum recorded sizes of 8 x 8 x 5, 27 x 27 x 16, and 20 x 19 x 19 mm. at the time of their respective autopsies, 77, 84, and 149 days after injection. All were diagnosed sarcoma. One mouse was negative at autopsy at 149 days.

Group 3.—Four mice were irradiated as described in experiment 2, and one whole culture was injected into each. One showed a tumor at 50 days which measured 10 x 9 x 6 mm. when found. The mouse was autopsied the same day. This tumor was diagnosed sarcoma. Three mice were negative at autopsy, 148 days after injection.

Group 4.—Each of four irradiated mice received one whole culture. Three mice showed tumors at 14, 64, and 149 days, which at autopsy 14, 66, and 149 days, measured 6 x 5 x 2, 25 x 22 x 20, and 10 x 10 x 10 mm., respectively. All were diagnosed sarcoma; the second tumor appeared to be regressing, as determined from a microscopic section. One mouse died at 115 days and was grossly negative.

Experiment 5

Cultures from strain J controls and strain N controls, and strains J and N carcinogen-treated cells were injected (July 3, 1942) 178 days after strain J, and 147 days after strain N was removed from carcinogen. All injections were made with syringes into male mice. This experiment included four groups:

Group JC.—Fourteen mice were injected with strain J control cultures. Twelve mice showed tumors 20 to 114 days after injection, most of the tumors appearing in about 60 to 80 days. The largest tumor showed a maximum recorded size of 45 x 21 x 20 at 81 days, and the smallest 3 x 3 x 3 mm. also at 81 days. One mouse, with a tumor 22 x 15 x 15 mm., died at 66 days and was not autopsied; three were autopsied at 103 to 134 days and had tumors diagnosed regressing sarcoma; eight autopsied at 63 to 134 days had tumors diagnosed sarcoma; two were killed at 134 days and showed no tumors.

Group NC.—Five mice were injected with strain N control cultures. Three mice showed tumors at 20, 49, and 134 days, respectively. The tumors measured 25 x 20 x 10, 25 x 20 x 3, and 7 x 5 x 2 mm., when the mice were autopsied at 70, 70, and 134 days. All tumors were diagnosed sarcoma. The other two mice when autopsied at 134 days were negative.

Group J.—Four mice were injected with strain J carcinogen-treated cultures. Two mice showed tumors at 53 and 114 days. The tumors reached maximum recorded sizes of 34 x 26 x 21, and 24 x 24 x 15 mm. at 64 and 118 days, respectively. The first of these two mice died at 64 days and

was not autopsied; the second was autopsied at 118 days, when the tumor was diagnosed sarcoma. The other two mice were autopsied at 134 days and were grossly negative.

Group N.—Thirty-one mice were injected with strain N carcinogen-treated cultures. Four showed tumors at 69 days, and three were autopsied at that time. The tumor sizes ranged from 12 x 10 x 10 to 20 x 15 x 15 mm. The fourth mouse, which was autopsied at 134 days, showed a tumor 22 x 17 x 10 mm. All tumors were diagnosed sarcomas. The other 27 mice were autopsied at 134 days and were negative.

Experiment 6

Injections of carcinogen-treated cultures from the H strain were made (July 17,1942) 218 days after the cultures were removed from the carcinogen. All injections were made by syringe into the back, and each mouse received one culture. This experiment consisted of two groups. Some injections in each group were made intramuscularly and some intraperitoneally, approximately the same relative number of each injection type being used in each group. Since the injection site seemed to have little influence on the results, it is left out of consideration.

Group 1.—Fifteen mice were used in this group. The culture mass was drawn up into the syringe through the needle. Thirteen of the mice showed tumors in 10 days and when autopsied at 28 to 33 days, it was found that the tumors ranged from 15 x 15 x 12 to 37 x 35 x 35 mm. in size. Two other tumors appeared at 88 days, and at autopsy at 104 days they were about 25 x 18 x 18 mm. in size. All the tumors were diagnosed sarcoma. There were no mice in the group which did not show tumors. Figure 1 shows five mice from this group.

Group 2.—Eleven mice were used in group 2. The culture mass was repeatedly sucked into the syringe through the needle and then ejected, until the clumps were thoroughly broken up and emulsified. Five of the mice injected showed tumors in 28 days or less, one in 55 days, and one in 88 days. One tumor-bearing mouse was lost at 104 days; six were autopsied at from 56 to 104 days and had tumors ranging from 8 x 8 x 7 mm. (56 days) to 35 x 30 x 25 mm. Six tumors were sectioned and

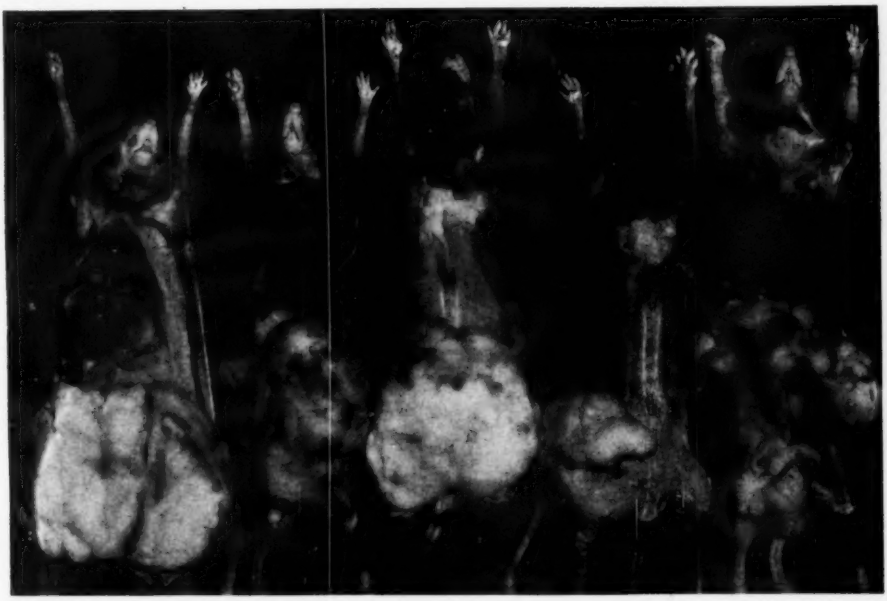

FIGURE 1.—Five representative tumor-bearing mice. The tumors arose from injection of strain H carcinogen-treated cells (experiment 6, group 1). The mice were autopsied 33 days after first inoculation.

all were diagnosed sarcoma. Four mice showed no tumor when autopsied at 104 days.

Experiment 7

Thirty-four male mice were injected (August 31, 1942) with strain L control cultures. One culture was injected into each mouse with a syringe which was loaded by funnel. Injection was made into the lumbar muscles and was rather deep so that in some mice it was probably retroperitoneal. Thirty mice showed tumors in from 22 to 79 days, the larger number appearing about 45 days. When the mice were autopsied at from 44 to 75 days, the tumors varied in size from 8 x 6 x 6 to 30 x 25 x 20 mm. Twenty-eight were diagnosed sarcoma, of which four seemed to be regressing. Two tumor-bearing mice were not autopsied; four mice were negative.

Experiment 8

Thirty male mice were inoculated by syringe (September 9, 1942) with one culture each from strain H control cultures; the injection was placed in the left groin and lumbar region. One mouse showed a tumor at 52 days; and at autopsy at 92 days, the tumor measured 22 x 15 x 15 mm. On sectioning it was diagnosed sarcoma. Twenty-nine mice were negative when autopsied at 126 days.

Experiment 9

Cultures from strain H controls, and H, J, L, and N carcinogen-treated cultures were injected (October 3, 1942) 296, 270, 191, and 239 days, respectively, after strains H, J, L, and N had been removed from carcinogen. All injections were made by syringes loaded through funnels, into the dorsal lumbar muscles of male mice, one culture for each mouse. There were five groups in this experiment.

Group HC.—Nine mice were each injected with one strain H control culture. Two mice showed tumors in 71 and 78 days, the respective tumors measuring 25 x 20 x 20 and 15 x 12 x 10 mm. at 102 days. Both were diagnosed sarcoma. Seven mice were negative when autopsied at 102 days.

Group H.—Ten mice were each injected with one strain H carcinogen-treated culture. One showed a tumor in 8 days, one in 36, and one in 88. When the three mice were autopsied at 36, 102, and 102 days, their tumors measured 32 x 20 x 20, 39 x 34 x 30, and 20 x 17 x 17 mm. All were diagnosed sarcoma. Seven mice were negative when autopsied at 102 days.

Group J.—Ten mice were each injected with one strain J carcinogen-treated culture. Two mice had tumors at 8 days; the tumors reached a size of 34 x 30 x 30 and 28 x 28 x 25 mm. at 71 and 64 days, respectively, at which time the animals were autopsied. Both tumors were diagnosed sarcoma. Eight mice showed no tumor at 102 days.

Group L.—The 10 mice in group L were each injected with 1 strain L carcinogen-treated culture. Three showed tumors at 22 days, and at 22 to 36 days the tumors reached a size of about 32 x 22 x 19 mm. Two were not autopsied; one was autopsied and sectioned, the tumor being diagnosed sarcoma. Six mice showed no tumors at 102 days.

Group N.—Nine mice were each injected with one strain N carcinogen-treated culture. One mouse showed a tumor in 8 days which reached a size of 25 x 20 x 20 mm. at 64 days, and at autopsy was diagnosed sarcoma. Eight mice showed no tumors at 102 days.

Experiment 10

Cultures from strain D controls and H, J, L, N, and O carcinogen-treated strains were injected (November 2, 1942) 326, 300, 221, 269, and 47 days, respectively, after strains H, J, L, N, and O had been removed from the carcinogen. All injections were made into the dorsal lumbar and thigh muscles of male mice, one culture to each mouse. The syringes were loaded through funnels. The experiment included six groups:

Group DC.—Four mice were each injected with one strain D control culture. Three showed tumors in from 11 to 30 days; the tumors reached sizes up to 20 x 18 x 15 mm. at 34 days, and at autopsy when sectioned were diagnosed sarcoma.

One mouse showed a tumor at 48 days which reached a size 36 x 27 x 20 mm. at 83 days. This mouse was not autopsied.

Group H.—Six mice were each injected with one strain H carcinogen-treated culture. Five showed tumors in 6 to 11 days, which at 20 to 39 days had reached sizes ranging from 19 x 7 x 7 to 30 x 24 x 24 mm. At autopsy at 20 to 39 days, all were diagnosed sarcoma. One mouse was negative at 103 days.

Group J.—Seven mice were each injected with one strain J carcinogen-treated culture. All were negative at autopsy 103 days after injection.

Group L.—Seven mice were each injected with one strain L carcinogen-treated culture. Four showed tumors at 11 to 18, and one at 48 days after injection. The recorded sizes of the tumors ranged from 13 x 12 x 10 to 30 x 30 x 30 mm. at 29 to 80 days. Four mice were autopsied at 29 to 40 days, and the tumors diagnosed sarcoma; one mouse bearing a tumor 30 x 30 x 30 mm. died at 80 days but was not autopsied. Two mice were negative when autopsied at 103 days.

Group N.—Seven mice were each injected with one strain N carcinogen-treated culture, and all were negative when killed 103 days after injection.

Group O.—Five mice were each injected with one strain O carcinogen-treated culture. One mouse at 48 days showed a tumor which enlarged to 19 x 16 x 16 mm. at 97 days. The mouse died but was not autopsied. Four mice were negative at 103 days.

Experiment 11

Cultures from strain D controls, and H, J, L, N, and O carcinogen-treated strains were injected (November 4, 1942) 328, 302, 223, 271, and 49 days, respectively, after strains H, J, L, N, and O had been removed from carcinogen. All injections were either of seven-eighths of a culture or one whole culture each and were made from syringes loaded through funnels. Where only seven-eighths of a culture was used, the culture was very large so that all mice can for practical purposes be considered as having received a whole culture each. Injections were made into dorsal groin and lumbar region of male mice. The experiment included six groups:

Group DC.—Five mice were injected with strain D control cultures, and all developed tumors, four of them in 37 days and the fifth in 67 days. They were autopsied at 56 to 100 days, and at autopsy the tumors ranged from 26 x 17 x 17 to 35 x 30 x 30 mm. All were diagnosed sarcoma.

Group H.—Seven mice were injected with strain H carcinogen-treated cultures. Five mice showed tumors in 9 days, one other in 18 days, while one was negative at 100 days. The six tumor-bearing mice were autopsied at 18 and 46 days when the tumor sizes ranged from 22 x 12 x 10 to 30 x 20 x 20 mm. All were diagnosed sarcoma.

Group J.—Seven mice were injected with strain J carcinogen-treated cultures. One mouse that developed a tumor at 56 days was autopsied at 100 days; the tumor measured 36 x 32 x 20 mm. and was diagnosed sarcoma. The other six mice were negative at 100 days.

Group L.—Seven mice were injected with strain L carcinogen-treated cultures. Three developed tumors in 9 days, and at autopsy 37 to 70 days after injection the tumors ranged from 27 x 19 x 19 to 36 x 30 x 30 mm. All were diagnosed sarcoma.

Group N.—Five mice were injected with strain N carcinogen-treated cultures. All mice were negative when autopsied at 100 days.

Group O.—Four mice were injected with strain O carcinogen-treated cultures. One showed a tumor in 9 days that enlarged to 13 x 10 x 10 mm. at 37 days. The mouse was autopsied at this time and the tumor diagnosed sarcoma. The other three mice were grossly negative at 100 days.

Experiment 11a

Experiment 11a was carried on in conjunction with experiment 11. Cultures used in experiment 11 to inoculate mice Nos. 2 (with strain D control cells), 9 (with strain H carcinogen-treated cells), 18 and 20 (with strain J carcinogen-treated cells), 27 (with strain L carcinogen-treated cells), 35 (with strain N carcinogen-treated cells), and 49 (with strain O carcinogen-treated cells) were employed. A uniform segment of less than one-eighth the circumference of each culture was removed, and the segments were inoculated separately into mice Nos. 1, 8, 17, 19, 26, 34, and 48, respectively. The parent cultures were also inoculated as described in experiment 11. Large parent cultures were selected so that the removal of one-

eighth of each did not reduce the size to less than that normal to cultures of the several strains. Each parent culture was used as a control for the one-eighth-sector culture taken from it.

The mouse injected with the seven-eighths culture of strain D showed a tumor in 37 days which reached a size of 35 x 30 x 30 mm. at 56 days. The tumor from the strain D one-eighth culture appeared in 37 days but reached a size of only 25 x 17 x 17 mm. in 56 days. Both tumors were diagnosed sarcoma.

The mouse injected with the seven-eighths culture of strain H developed a tumor in 9 days that measured 23 x 18 x 18 mm. at 18 days. The tumor from the one-eighth injection also appeared in 9 days and reached a size of 35 x 25 x 25 at 27 days. Both tumors were diagnosed sarcoma.

All injections, either of seven-eighths or of one-eighth clump from strains J, L, N, and O, were negative at 100 days.

Experiment 12

Cultures from strain D controls, and H, J, L, N, and O carcinogen-treated strains were injected (November 10, 1942) 334, 308, 229, 277, and 55 days, respectively, after strains H, J, L, N, and O had been removed from carcinogen. All injections were of one culture each and were made with syringes loaded through funnels. The injections were made into the dorsal groin and lumbar region. The experiment included six groups of mice:

Group DC.—Six mice were each injected with one D control culture. Four developed tumors in 12 to 31 days, the other two at 61 days. The tumors ranged in size from 25 x 16 x 12 to 30 x 25 x 20 mm. at 40 to 94 days. Four mice were autopsied and the tumors diagnosed sarcoma; two mice bearing large tumors died but were not autopsied.

Group H.—Seven mice were each injected with one strain H carcinogen-treated culture. Five developed tumors at 12 days which reached sizes from 20 x 15 x 15 to 38 x 23 x 20 mm. at 29 days. All tumors were diagnosed sarcoma. Two mice were negative at 94 days.

Group J.—Seven mice were each injected with one strain J carcinogen-treated culture. One mouse developed a tumor at 40 days which measured 13 x 13 x 13 mm. at 94 days and was diagnosed sarcoma. The other six mice were negative at 94 days.

Group L.—Five mice were each injected with one strain L carcinogen-treated culture. Three had tumors at 40 days; autopsy at 50 days showed that the tumors ranged from 20 x 15 x 10 to 35 x 30 x 30 mm. Two tumors were sectioned and diagnosed sarcoma. The third mouse was not autopsied. The two remaining mice were negative at 94 days.

Group N.—Five mice were each injected with one strain N carcinogen-treated culture, and all were negative at 94 days.

Group O.—Eight mice were each injected with one strain O carcinogen-treated culture, and all were negative at 94 days.

Experiment 13

Cultures from strain D controls, and H, J, L, N, and O carcinogen-treated strains were injected (November 11, 1942) 335, 309, 230, 278, and 56 days, respectively, after strains H, J, L, N, and O had been removed from carcinogen. All injections were made with syringes loaded through funnels with one culture each and into dorsal hip and lumbar region. The experiment included 6 groups of mice:

Group DC.—Ten mice were each injected with one strain D control culture. One mouse developed a tumor in 11 days which had grown to 29 x 16 x 16 mm. at 49 days. Four developed tumors at 39 to 49 days, and one at 69 and one at 88 days. Five tumor-bearing mice were autopsied at 49 to 93 days after injection; the tumors ranged from 22 x 15 x 10 to 28 x 28 x 20 mm. and were diagnosed sarcoma. Two mice bearing large tumors died at 73 and 80 days but were not autopsied. Three mice were normal at 93 days.

Group H.—Nine mice each received an injection of one strain H carcinogen-treated culture. Six developed tumors at 11 days which at 19 to 49 days reached sizes from 20 x 15 x 10 to 36 x 26 x 26 mm. The six mice were autopsied at 19 to 49

days and all tumors were diagnosed sarcoma. The other three mice were grossly negative at 93 days.

Group J.—Ten mice were each injected with one strain J carcinogen-treated culture. Three developed tumors in 11, 30, and 60 days; the first two were autopsied at 49 and 93 days, and the tumors were found to measure 25 x 20 x 20 and 39 x 26 x 26 mm., respectively. The smaller tumor was sectioned and diagnosed sarcoma. The other seven mice were negative at 93 days.

Group L.—Twelve mice each received an injection of one strain L carcinogen-treated culture. One mouse developed a tumor in 11 days which at 49 days reached a size of 30 x 25 x 25 mm. and was diagnosed sarcoma. The other 11 were negative at 93 days.

Group N.—Nine mice were each injected with one strain N carcinogen-treated culture. All were negative at 93 days.

Group O.—Nine mice were each injected with one strain O carcinogen-treated culture. All were negative at 93 days.

Experiment 14

Cultures from strain D controls, and H, J, N, and O carcinogen-treated strains were injected (December 1, 1942) 355, 329, 298, and 76 days, respectively, after strains H, J, N, and O had been removed from carcinogen. Instead of injecting one whole culture into each mouse, two strips were cut from each O strain culture of such size and shape that they included the mass of living cells. The strips were trimmed to uniform size, and then strips of the same size were cut from the growing areas of cultures of the other strains. This procedure gave two strips of equal area from the growing zone of all cultures. In the case of the more luxuriantly growing cultures, care was taken to cut the strips from the less densely cellular areas so that the cell density in all strips was roughly comparable, the N and O strain strips, if anything, had greater cell density than the D and H strain strips. Each pair of strips from one culture was injected into one mouse. All injections were made with syringes loaded through funnels and into the dorsal hip and lumbar region. The experiment included five groups of mice:

Group DC.—Twelve mice were each injected with strips from a strain D control culture. Ten showed tumors in 17 to 38 days, and at 44 to 51 days the tumors ranged from 16 x 10 x 10 to 39 x 28 x 28 mm. Eight mice were autopsied at 54 to 63 days; seven tumors were sectioned and diagnosed sarcoma. Two mice bearing large tumors died but were not autopsied. The other two mice were negative at 106 days.

Group H.—Twelve mice were each injected with one strain H carcinogen-treated culture. Eight showed tumors at 12, and one at 17 days. The tumor sizes ranged from 10 x 10 x 10 at 37 days to 30 x 25 x 25 mm. at 38 days. All these tumors were diagnosed sarcoma. The other three mice were negative at 106 days.

Group J.—Twelve mice were each injected with one strain J carcinogen-treated culture, and all were normal at 106 days.

Group N.—Eleven mice, which were injected with one strain N carcinogen-treated culture each, were normal at 106 days.

Group O.—Nine mice received an injection of one strain O carcinogen-treated culture each. Two mice developed tumors at 12 and 49 days, which measured 25 x 17 x 17 and 32 x 22 x 20 mm. at 54 and 102 days, respectively. Both tumors were diagnosed sarcoma. The other seven mice were negative at 106 days.

Subinoculations of Tumors Originating From Inoculations of Different Cell Strains

The various tumor strains started from the original inoculation of the control and the carcinogen-treated cells of the different strains may be summarized as follows:

Strain D control cells gave rise to one tumor strain which at present is in its fourteenth tumor generation.

Strain H control cells also gave rise to one tumor strain, at present in its fourteenth tumor generation.

Strain J control cells gave rise to a tumor strain that was carried three tumor generations and dropped.

Strain H carcinogen-treated cells gave rise to two tumor strains, the first of which was carried five tumor generations and then dropped while

the second is now in its fourteenth tumor generation.

Strain J carcinogen-treated cells gave rise to a tumor strain now in its fourteenth tumor generation.

Strain L carcinogen-treated cells gave rise to two tumor strains; the first was carried through seven tumor generations and then dropped, and the second is now in its fourteenth tumor generation.

Strain N carcinogen-treated cells gave rise to three tumor strains: the first grew poorly and failed to grow at all in its third generation; the second was carried through seven tumor generations and dropped; and the third is now in its fourteenth tumor generation.

Strain O carcinogen-treated cells gave rise to one tumor strain now in its fourteenth tumor generation.

On injection into strain C3H mice, tumors from these various strains regularly appeared as easily palpable masses in 5 to 10 days. As with all tumor inoculations, an occasional generation of a tumor strain was poor, probably owing to selection of more or less necrotic tissue for transfer; but with the exception of a few instances of this kind, the growth of the tumors was rapid and regular, and tumors appeared in nearly 100 percent of the injected mice.

During the earlier generation of these tumor strains, it was noted that different strains seemed to have different growth rates. The study of these growth rates is now in progress and will be reported later.

Metastases

The production of metastases is considered in the next paper (*2*). Metastases to the lungs were observed in five mice injected with strain L carcinogen-treated cultures, and one instance was noted in tumors arising from strain O carcinogen-treated cultures.

DISCUSSION

The whole group of culture strains in these experiments demonstrated a closely graduated morphologic transition from the H control strain (HW), which was barely perceptibly altered morphologically from the normal cells of origin, to the most severely altered cells of strain O which had been subjected to methylcholanthrene for 406 days.

Experiments 1 through 14 show that when cells of each of these culture strains were inoculated into mice of the C3H strain, a tumor developed rapidly at the site of injection in a certain number of the mice. The tumor increased in size and usually killed the mouse unless its course was interrupted by autopsy. Microscopic study of all the tumors examined uniformly showed a structure typical of sarcoma, such as arises from fibroblast or mesenchymelike cells of the connective tissues. When culture strains had been treated with carcinogen for long intervals, some mice showed definite metastases to the lungs. Subinoculations have been successful with tumors which arose from each of the cell strains injected.

From these data and from the more detailed microscopic examination (*2*), there seems no doubt that these tumors must be classed as malignant, as sarcomas, and as being readily transplantable into the strain of mice from which the primary cell strain was originally taken.

Bryan and Shimkin (*4*) reported that the injection of even 1,000γ of methylcholanthrene in tricaprylin into strain C3H mice could induce tumors in only 1 percent of the mice in about 40 days. In the instance of production of tumors by injection of the cell strains described herein, the time required for the production of many of the tumors to a stage where they were easily palpable and grew steadily was much less than 45 days, whereas some strains fairly consistently gave such tumors in 9 to 12 days. These data appear conclusive that the tumors developing after injection of the cell clumps did not arise

because of the action of injected carcinogen but from the proliferation of the injected cell masses. This conclusion is confirmed by the fact that several sections obtained from early tumor stages gave histologic evidence that the tumors were arising from the cells of the injected cell mass.

In view of the gradual, morphologic alterations of the cells and the production of tumors by those cells, there seems little doubt that apparently normal cells taken from an apparently normal and healthy young C3H mouse and grown in a completely heterologous culture medium entirely removed from the systemic reactions of the original parent host and subjected to the action of a chemical carcinogen, 20-methylcholanthrene, undergo progressive morphologic alteration. When so altered, they can give rise to sarcomas on reinjection into C3H mice. That this ability to undergo such alteration is not confined to cells of the C3H mouse in the presence of methylcholanthrene is shown by the work of Jacoby (*5*), who described a similarly altered architecture of tissue cultures from tumors induced in rats by injection of 1, 2, 5, 6-dibenzanthracene.

The injection data in experiments 1 to 14 were obtained under various experimental conditions. If certain of the experiments and experimental groups are eliminated, a fairly comparable group of data is left. Experiments 1 and 2 must be eliminated entirely from consideration of the data, since in experiment 1 two cell types were injected and in both experiments 1 and 2 trocars were used to inject the cell mass, with possibly increased hazard of drying and of loss of cells. Similarly, injections of irradiated mice and of less than a whole culture must also be disregarded, with the exception of experiment 11 in which the injection of seven-eighths of a culture was made. The final group of data is summarized in table 1.

TABLE 1.—*Tumors produced on injection of cells of different cell strains*

Cell strain	Number of injections	Percentage
H control	39	7.7
D control (and other controls)	84	86
H	66	72
J	47	20
L	41	46
N	67	7.6
O	27	7.6

The question arises whether differences in the relative abilities of different culture strains to induce tumors, as shown in table 1, are due to the relative number of cells injected. Since the strain O cultures were extremely dense but covered only limited areas, the actual number of cells injected from a strain O culture was almost certainly less than that from a strain D control or a strain H carcinogen-treated culture, for instance. In experiment 14, however, the relative amounts of cells injected was altered so that the strain O injections had at least as large a number of cells as did injections from strain D controls or strain H carcinogen-treated cultures. In spite of this change, strain D control cultures gave rise to 10 tumors from 12 injections; strain H cultures 9 from 12 injections; strains J and N no tumors from 12 and 11 injections, respectively; while strain O gave rise to 2 tumors from 9 injections.

In experiment 11a a similar test was made, but less than one-eighth of a culture was used from each strain, the remaining seven-eighths being used as a control. In D control and H carcinogen-treated cultures, cell clumps containing either one-eighth or seven-eighths of a culture gave rise to tumors on injection, whereas in strains J, L, N, and O neither the one-eighth nor the seven-eighths of a cell mass did so. At this time, the relative number of cells in D controls and H carcinogen-treated cultures was certainly

not seven times as great as in strains J, L, N, and O carcinogen-treated cultures.

The conclusion from both of these experiments is that the difference in the frequency with which the inoculation of cultures of different strains gave rise to tumors resulted from a difference intrinsic in the cell strains rather than from a difference in the number of cells in the cultures inoculated. Accepting the working hypothesis that a trace contamination of methylcholanthrene had produced the morphologic changes observed in the control cultures, the conclusion is reached that the relative ability of these strains of cells (both control and experimental) to give rise to tumors is closely associated with the dose of carcinogen that the cells received and with the morphologic alteration induced.

The percentage of tumors obtained for each cell strain (table 1) was plotted against the time the cultures were exposed to 1γ of methylcholanthrene per cubic centimeter of culture fluid. The result is shown in figure 2.

Plotted in this way, while the positions of the points of strains H, J, L, N, and O are fixed along the horizontal axis, the position of strains HW (H control) and D (D control) are not fixed. From the morphologic changes observed in the cells (*1*), the position of the points for both strains D control (D) and H control (HW) can be fixed along the abscissa as definitely to the left of point H, H to the left of point D, and both to the right of zero. Point D is already so close to zero that we can tentatively fix the horizontal position of this point as halfway between zero and H, and point HW as halfway between zero and point D along the horizontal axis. This was done in figure 2, where dotted lines connect points HW and D with H.

If the shape of this curve is approximately correct, certain conclusions are indicated from it:

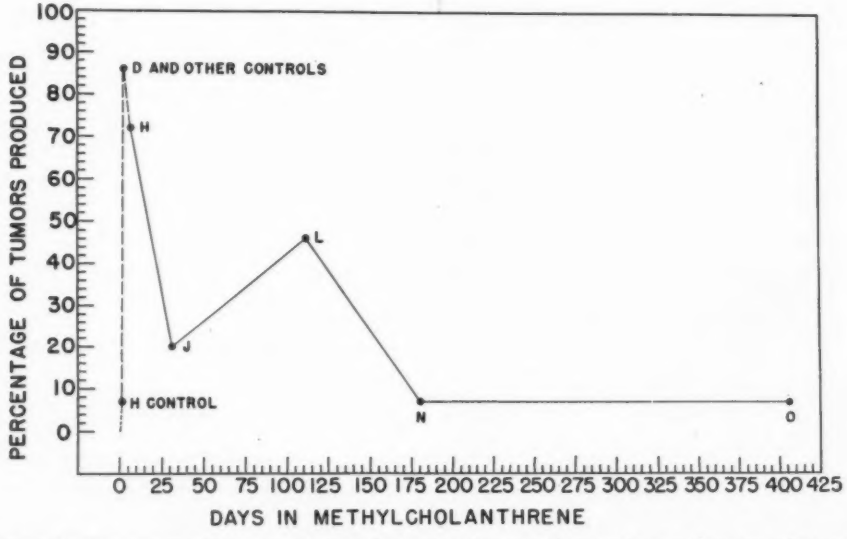

FIGURE 2.—Percentage of tumors obtained for each cell strain (ordinate) as a function of the time the cell strains were subjected to a 1γ concentration of methylcholanthrene in days (abscissa).

(1) Since a relatively slight shift to the left along the horizontal axis from point D control to point H control (HW) resulted in a drop in tumor production from 86.0 to 7.7 percent and since these strain HW control cultures were recognizably morphologically altered, it seems most unlikely that the original primary strain of cultures, before this alteration, had the power of giving rise to sarcoma. If this is true, the left end of the curve would pass through the zero point on abscissa and ordinate, and it has been so drawn in figure 2.

(2) The horizontal distance from the probable origin of the curve to point H control (HW), a point of very low tumor productivity, is very slight, while the distance from that point to point O is much greater. Certainly, too, the morphologic change from the normal cell to one capable of inducing a tumor is very slight indeed as compared with the further change that the cell can undergo on continued exposure to carcinogen before it reaches a stage of morphologic change so great that it can no longer live. The most interesting point for speculation is whether the primary physiologic change from the normal cell to one capable of producing sarcoma is not similarly minute as compared with the further progressive change the cell can undergo through continued treatment with carcinogen without dying.

(3) The point of maximum percentage of tumor production from primary injections appears definitely less than that seen in cells exposed to 1γ of methylcholanthrene per cubic centimeter of culture fluid for 6 days. How much less cannot be estimated. This conclusion, together with the other data presented, emphasizes the importance of the study of cells over an extended interval with lower carcinogen dosage than that used in the present work.

When the rate of increase of width in the cell clump is plotted against the days in methylcholanthrene (*1, fig. 22*), there is a sharp drop at the point for strain J, a definite rise for strain L, and then a final drop for strains N and O. As depicted in figure 2 of the present paper, the curve for the percentage of tumors produced, as a function of the number of days in the carcinogen, shows the same relationship for the points for strains H, J, L, N, and O. The accuracy of width measurements of the culture width curves might conceivably be such that point J was too low and point L too high, so that a correction of the positions of these points might give a smoothly rounded curve from point H down to point N. When, however, this same characteristic irregularity in shape appears in the curve of the percentage of tumors produced, a curve derived from a totally different type of measurement, it cannot be dismissed as the result of such possible error. Nor is there any possibility that strains J and L could have been interchanged, since the degree of morphologic cell change in the cultures of strain J has been and was in May 1943 perceptibly less than that in the cultures of strain L. It is, therefore, apparent that this seeming irregularity in the relative height of points J and L on these two curves is a real phenomenon, although at the present time data available do not allow further interpretation of its cause or nature.

The question naturally arises why Earle and Voegtlin (*6*) did not obtain tumors in the 12 mice they injected with the cultures they identify as strain 188. These cultures were injected 83 days after removal from a concentration of 10γ of methylcholanthrene per cubic centimeter of culture fluid in which they had been held for 114 days. Since, as these authors showed (*6, 7*), the action of the carcinogen increases with its concentration, the likelihood is that the cells of strain 188 were comparable with about strain N (184 days at 1γ) in the

present research. This is also indicated by the observation that the change in the appearance of the cells and the cultures in strain 188 was approximately comparable with that shown in strain N of the present study but certainly not so severe as that in strain O. The injected cultures of strain N produced about 7.6 percent tumors, or about 1 tumor in 13 injections. It is unfortunate that only 12 injections of the cultures of strain 188 were made and that the work was then interrupted, as the present data suggest that a longer series of injections would probably have given rise to tumors.

SUMMARY AND CONCLUSIONS

Data are presented showing the results of injection of seven strains of fibroblast cultures into young strain C3H mice. These strains had all arisen from a primary strain of fibroblasts, from a C3H mouse, grown in vitro in a mixture of horse serum and chick-embryo extract. The strains included two control strains, one of which was very slightly altered morphologically (H control) and the other more altered (D control). The alteration is considered as most probably having resulted from trace contamination of the cultures with 20-methylcholanthrene, although the possibility of its having arisen spontaneously cannot as yet be ruled out. The experimental strains were all subjected to the carcinogen in a concentration of 1γ per cubic centimeter of culture medium for different periods. Strain H was subjected for 6 days and was definitely more altered than strain D control; strain J was subjected for 32 days, strain L 111 days, strain N 184 days, and strain O 406 days, after which they were removed from carcinogen for various periods and then injected.

Cultures of each cell strain injected gave rise to tumors in the following percentage of injections: H control 7.7; D control, 86; H carcinogen-treated, 72; J carcinogen-treated, 20; L carcinogen-treated, 46; N carcinogen-treated, 7.6; and O carcinogen-treated, 7.6.

Because of the gradual morphologic alteration of the cells under the action of carcinogen and of the production of tumors by these altered cells, it seems clear that apparently normal fibroblasts taken from an apparently normal, healthy, young C3H mouse and grown in a completely heterologous culture medium entirely removed from the systemic reactions of the original host are progressively altered morphologically when exposed to the action of a chemical carcinogen, 20-methylcholanthrene. When so altered they can give rise to sarcomas on reinjection into C3H mice. The very low percentage of tumors produced by strain HW cells (H control), only very slightly altered morphologically, suggests that the normal fibroblast culture, characterized by its usual loose architecture, can produce few or no tumors on injection.

The difference in frequency with which the inoculations of cultures of different strains produced tumors arose from a difference intrinsic in the cell strains and not from a difference in the relative numbers of cells in the cultures inoculated.

If it is accepted that a trace contamination of methylcholanthrene had produced the morphologic changes observed in the control cultures, the conclusion is reached that the relative ability of the cell strains to give rise to tumors on injection is closely correlated with the dose of carcinogen the cells received and with the morphologic alteration induced in the cells by the carcinogen.

The sarcomas produced by injection of cells of the different strains grew rapidly, and unless their course was interrupted by autopsy they usually caused the death of

the host mouse. They were readily transplantable, and frequently metastasized when derived from cultures exposed to the carcinogen as long as 111 days.

When a concentration of 1γ of methylcholanthrene per cubic centimeter of culture fluid is used, maximum tumor production probably occurs from the injection of cells exposed to the carcinogen less than 6 days, while continuous exposure for as long as 184 to 406 days alters cells so greatly that they can less readily produce tumors on inoculation. It is emphasized that further work should be done on the action of methycholanthrene in doses of less than 1γ for 6 days.

REFERENCES

(1) EARLE, W. R.: Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst., 4: 165–212 (1943).

(2) NETTLESHIP, A., and EARLE, W. R.: Production of malignancy in vitro. VI. Pathology of tumors produced. J. Nat. Cancer Inst., 4: 229–248 (1943).

(3) EARLE, W. R., and VOEGTLIN, C.: A comparison of the growth of the Jensen rat sarcoma in subcutaneous and intramuscular transplants. Nat. Inst. Health Bull. No. 164 (1935).

(4) BRYAN, W. R., and SHIMKIN, M. B.: Quantitative analysis of dose-response data obtained with three carcinogenic hydrocarbons in strain C3H male mice. J. Nat. Cancer Inst., 3: 503–531 (1943).

(5) JACOBY, F.: Architecture of colonies of a pure strain of fibroblastic sarcomatous cells derived from a dibenzanthracene mouse tumour. (Letter to editor.) Nature, London, 146: 301–302 (1940).

(6) EARLE, W. R., and VOEGTLIN, C.: A further study of the mode of action of methylcholanthrene on normal tissue cultures. Pub. Health Rep., 55: 303–322 (1940).

(7) EARLE, W. R., and VOEGTLIN, C.: The mode of action of methylcholanthrene on cultures of normal tissues. Am. J. Cancer, 34: 373–390 (1938).

548963—43——7

Production of Malignancy in Vitro. VI. Pathology of Tumors Produced

By ANDERSON NETTLESHIP, *passed assistant surgeon (R)*, and WILTON R. EARLE, *senior cytologist*, with the technical assistance of MARY P. CLAPP and EMMA SHELTON, *National Cancer Institute, National Institute of Health, United States Public Health Service*

The two preceding papers (*1*, *2*) describe how cells of a primary strain of fibroblasts were first isolated from the subcutaneous and fatty tissues of a young strain C3H male mouse and maintained in a heterologous culture medium. It is also shown how different groups of cultures of this primary strain were treated for various periods with a 1γ concentration of 20-methylcholanthrene per cubic centimeter of culture fluid and then reinjected into strain C3H mice. The method of injection of the cultures and the appearance of tumors at the injection sites have already been described (*1*, *2*). The present paper presents in detail the pathology of those tumors.

GROSS PATHOLOGY

The descriptions of the gross pathology are derived from a study of all tumor strains resulting from both the original implants and the first three generations of subinoculations. They are similar in all respects, except that the transplanted tumors tended to have more extensive necrosis.

On gross examination, the tumors were found growing at the site of inoculation, in or about the left kidney or back muscles on the left side, intraperitoneally in the left kidney region, or deep in the muscles of the left thigh and pelvis. In individual cases, the tumor presented itself as a rounded, hard, nodular mass firmly attached to the skeleton and muscles. All primary-inoculation tumors were hard. Subinoculations were carried out with each strain, and some of the resulting tumors were very soft. At autopsy, in either primary inoculations or subinoculations, the tumors varied considerably in size. Some that grew poorly or on which autopsies were performed early were as small as 3 mm. in diameter; others were enormous, measuring 45 x 30 x 30 mm., or larger. Though rarely adherent to the skin, a few tumors which had progressed to large size grew into the skin and ulcerated through the surface. Ordinarily, the tumors were not adherent, and the skin could be slipped off readily, revealing a well-rounded but not encapsulated, firm, yellowish-gray mass, the deeper portions of which had grown solidly into near-by muscles (fig. 1, *A*). Although no well-defined capsule was seen, the subcutaneous aspect of these tumors did not grow into surrounding structures. In contrast with this, the deep border invaded freely and destroyed muscles, kidney, bone, or any structure lying in its path. While the blood vessels adjoining the tumor were enlarged and dilated, usually the tumor proper was not overly vascular.

On section the tumors were rubbery and cut with some difficulty. Examination of the external surface of the tumor usually showed no evidence of necrosis; on gross section, however, necrosis was the invariable rule except in the tumors which had grown from primary-culture inoculation (fig. 1, *B*). The necrosis was central, spreading outward in an irregular, fan-shaped fashion, into the outer zone of firm, pink, viable tumor which was spared. The

FIGURE 1.—*A*, Strain L tumor resulting from original tissue-culture inoculation. The tumor has invaded from the left flank region and destroyed the left kidney, and occupies the lower portion of the abdomen. Approximately natural size; *B*, Cut surface of strain H tumor, slightly enlarged. The tumor has grown about the back muscles and onto the opposite flank. Abundant fresh necrosis is seen at the top of the tumor.

growing tumor edge where it had invaded muscle could not be defined. On section, the nonnecrotic portion of the tumor formed an irregular, gray-pink or translucent white, outer zone about 3 to 5 mm. wide. This area bulged; the necrotic areas were slightly depressed. The areas of necrosis proper varied in appearance. They ranged from fresh necrosis with tissue which was paler and softer than the live portion of the tumor, with a faint, thin edge of hemorrhage, to older necrosis with large, yellowish, caseous areas edged with bright red bands which grossly resembled fresh hemorrhage. The largest tumors showed large, irregular, necrotic

areas of all sorts, in some instances with almost total necrosis. Depending upon the extent of involvement of the thigh or back muscles, there was partial to total destruction of these muscle groups. Deeper implantation allowed the tumor to grow into the kidney or pelvis. In this case, the tumor grew into the pelvic cavity and about the left ureter, vagina, bladder, and rectum. It also invaded and destroyed pelvic bone. As the tumor advanced, all hollow viscera within the pelvis suffered from partial or total obstruction producing hydro-ureters, hydronephrosis, and megacolon. When the implant had been in the kidney, the tumor would destroy it; if it was implanted slightly below the lower pole of the kidney, it produced high obstruction by growing about the ureteropelvic junction. If the implantation had been intraperitoneal, widespread growth of the tumor over the viscera was encountered. The tumor grew into and about the coils of the small intestine, mesentery, and omentum, among the lobes of the pancreas, and into the capsule of the spleen.

It was difficult to distinguish which of these intraperitoneal tumors arose from fragments of the original injection and which were metastatic. Those cases in which the tumor was widespread showed multiple rounded masses ranging in size from 1 to 20 mm. and involving practically every organ in the abdominal cavity except the liver. One of the animals with multiple abdominal masses did show a tumor in the liver. In five cases, frank metastases occurred in the lungs and mediastinum. The metastases occurred only with the larger tumors and those showing the most extensive necrosis. Except for the questionable case of the tumor in the liver, all metastases were confined to the lung or associated mediastinal structures, these latter possibly by extension from the lung metastases. In the five cases of lung metastases, the tumors made their appearance more often in the left than in the right lung and were found to originate most often in the periphery of the lungs beneath the pleura. The growth, while extending into the pleura, did not cause adhesion between parietal and visceral pleurae. Grossly, the metastases were similar to the small nodules in the abdomen, gray-white, irregular in shape and did not show gross evidence of either necrosis or hemorrhage. The largest of the lung metastases measured 4 x 2 x 1 mm.

Other gross findings were anemia; emaciation, particularly in extensive peritoneal involvement; and splenomegaly. Two cases of lobular pneumonia and occasional spotted livers (mouse typhoid?) were encountered.

In all, 838 animals were examined at autopsy, including mice which had received the original subinoculation of tissue culture and also those which had received subinoculations of tumor tissue through the fourth generation of inoculation. Of these, 578 bore tumors. Complete autopsies were performed on all animals autopsied in the earlier portion of the study; later, brain examinations were omitted. After examination of the tumor and the organs involved, a thoracic and abdominal dissection was made, with examination of each organ.

HISTOPATHOLOGY

In the earlier phase of the study, all organs and the tumor were sectioned. The tumor section was taken in such fashion as to include a small amount of surrounding tissue, the outer viable zone of tumor, and a small segment of the necrotic areas. Later the sections were confined to the tumor and its extensions, heart and lungs, and any other questionable metastatic site.

After an animal was killed, the sections were obtained as rapidly as possible and fixed immediately in Zenker's acetic and/or Orth's fluid. The usual paraffin embedding technique was followed routinely with haematoxylin and eosin stains, and in selected cases special stains, such as Laidlaw's silver impregnation stain for reticulum, phosphotungstic acid-haematoxylin, Mallory's aniline blue, and the elastic collagen method of Verhoeff-van Gieson. Fifty percent of the tumors were examined microscopically.

Microscopically, these sarcomas are like the ones previously described by Haaland (*3*), Slye, Holmes, and Wells (*4*), and Heidenhain (*5*), as arising spontaneously in mice or produced by direct injection of a carcinogen into the subcutaneous region. Nor are they greatly different from the spindle-cell sarcoma or fibrosarcoma which occur in man. The majority of them were made up of large spindle-cell types (fig. 2, *A*).

Some tumors were composed of smaller spindle cells. Within a particular tumor the picture was fairly consistent, but from tumor to tumor there was great variability, and in some there was extreme variation in cell size, form, and nuclear structure. The spindle cells which made up the major portion of the tumor most closely resembled fusiform fibroblasts. The cells were generally larger than normal fibroblasts, in section about 75μ to 90μ long and 10μ to 12μ wide, and difficult to measure because of their closeness to other cells. Their cytoplasm was lightly granular and acidophilic. The nuclei were elongate, measuring about 8μ wide and 20μ to 25μ long, with gently curved ends, strongly stained periphery, and scattered, loose, granular karyoplasm. Each nucleus usually contained a single nucleolus. However, in some instances two or more nucleoli of small to moderate size were encountered. The fibroblastic nature of the tumor was further manifested by the formation of interlacing bundles (fig. 2, *B*), the intimate association of each cell with reticulum (fig. 3, *A*), and the presence of fibroglial fibers. In many areas the stroma was well-developed, as shown by Mallory's aniline blue stain (fig. 3, *B*). The commonest variation from the straight interlacing pattern of cells was the formation of fasciculi. This may have depended upon the regrowth of tumor back into a partially necrotic area following the formation of new granulation tissue. Remnants of the invaded tissue, such as muscle, fat, bone, or kidney were found among the sarcoma cells.

The fusiform neoplastic cells invaded the surrounding structures in broad sheets although some areas showed individual cells pushing into the stroma between normal cells. In areas of solid penetration, normal tissue was destroyed. Nerves were found which were almost completely atrophic; blood vessels which had been invaded were thrombosed. When the sarcoma cells invaded the skin, they pushed in about hair follicles through collagen and elastic fibers and destroyed them progressively towards the surface (fig. 4, *A*). In other areas the tumor cells actually invaded the epidermis and grew through it. In the bone the periosteum was first attacked, resulting in partial or total destruction; the invasion then proceeded directly, with cortical destruction and bone-marrow-cavity involvement (fig. 4, *B*). Muscle was infiltrated and destroyed by growth between and around the fibers, which were destroyed as the tumor progressed (fig. 5, *A*). The kidney was invaded from the capsule inward, and the tubules were destroyed. Glomeruli were surrounded, and the capsular spaces became filled with tumor, and capillary tufts were compressed and obliterated,

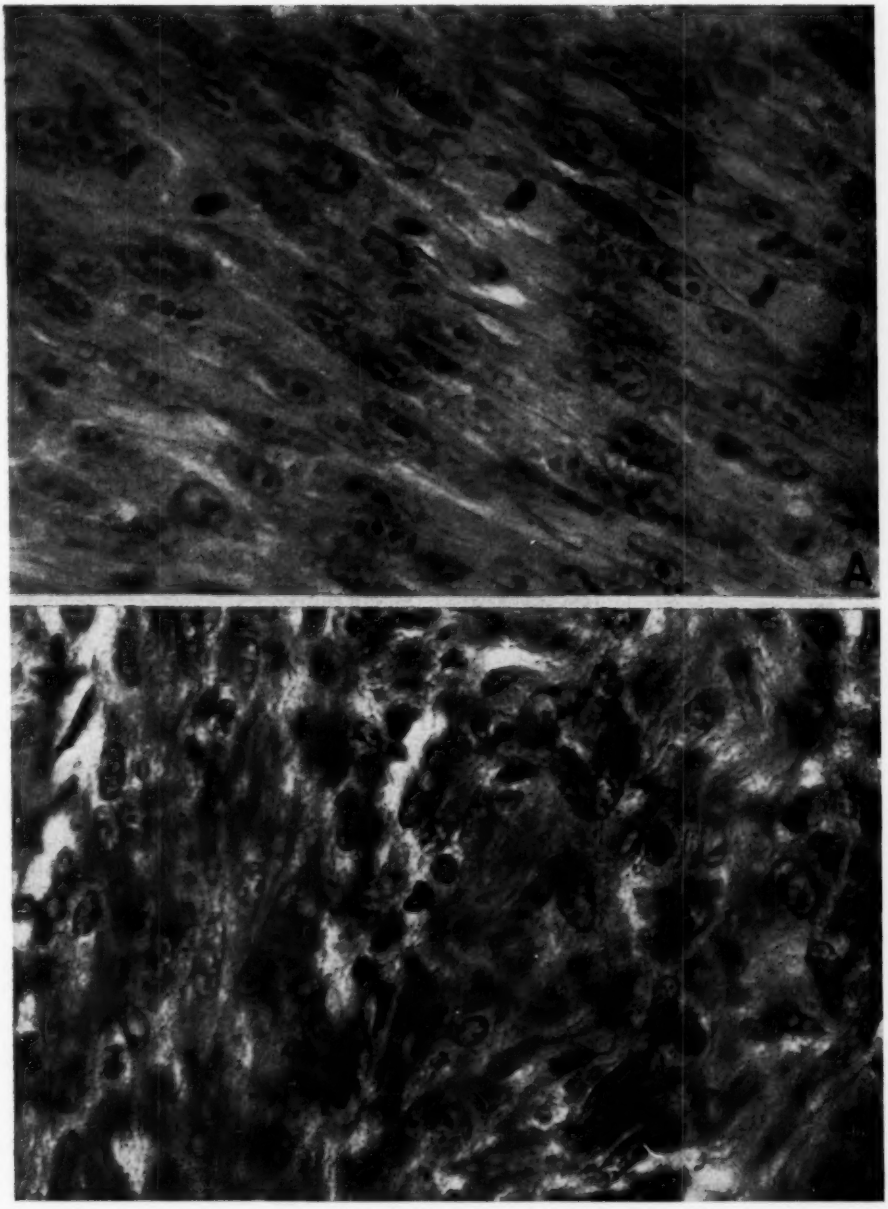

FIGURE 2.—*A*, Section from a primary N strain tumor showing the elongated spindle cells and regularity in cell pattern. The character of the cells and nuclei are shown, as are three mitoses. Verhoeff-van Gieson stain. × 1600; *B*, Section of an N strain sarcoma showing whorls and interlacing elongated cells. Phosphotungstic acid-haematoxylin stain. × 1600.

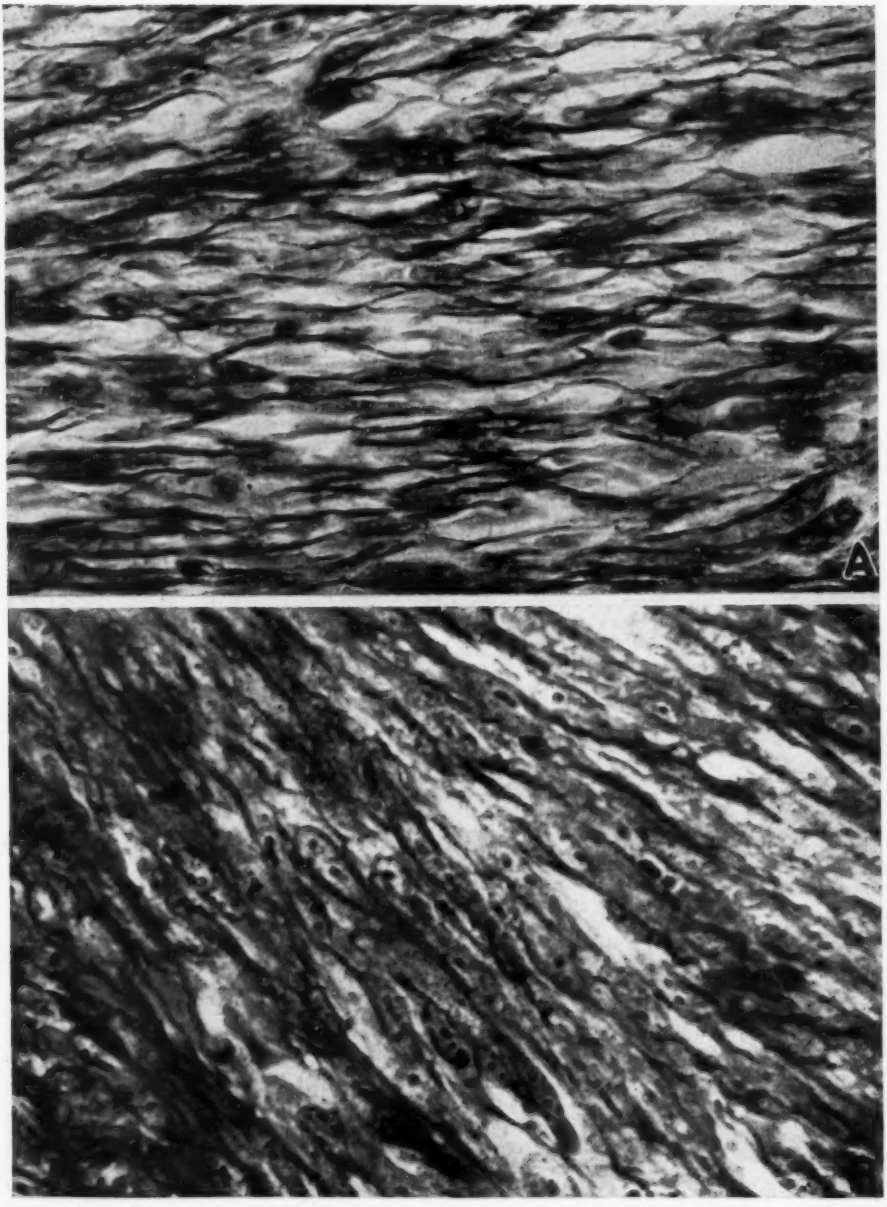

FIGURE 3.—*A*, Section from an N strain sarcoma taken through the longitudinal axis of the cells, showing how the reticulum fibers are split about single sarcoma cells. Laidlaw's reticulum stain. × 1600; *B*, Section from an N strain tumor showing well-developed stroma. Mallory's aniline blue stain. × 1600.

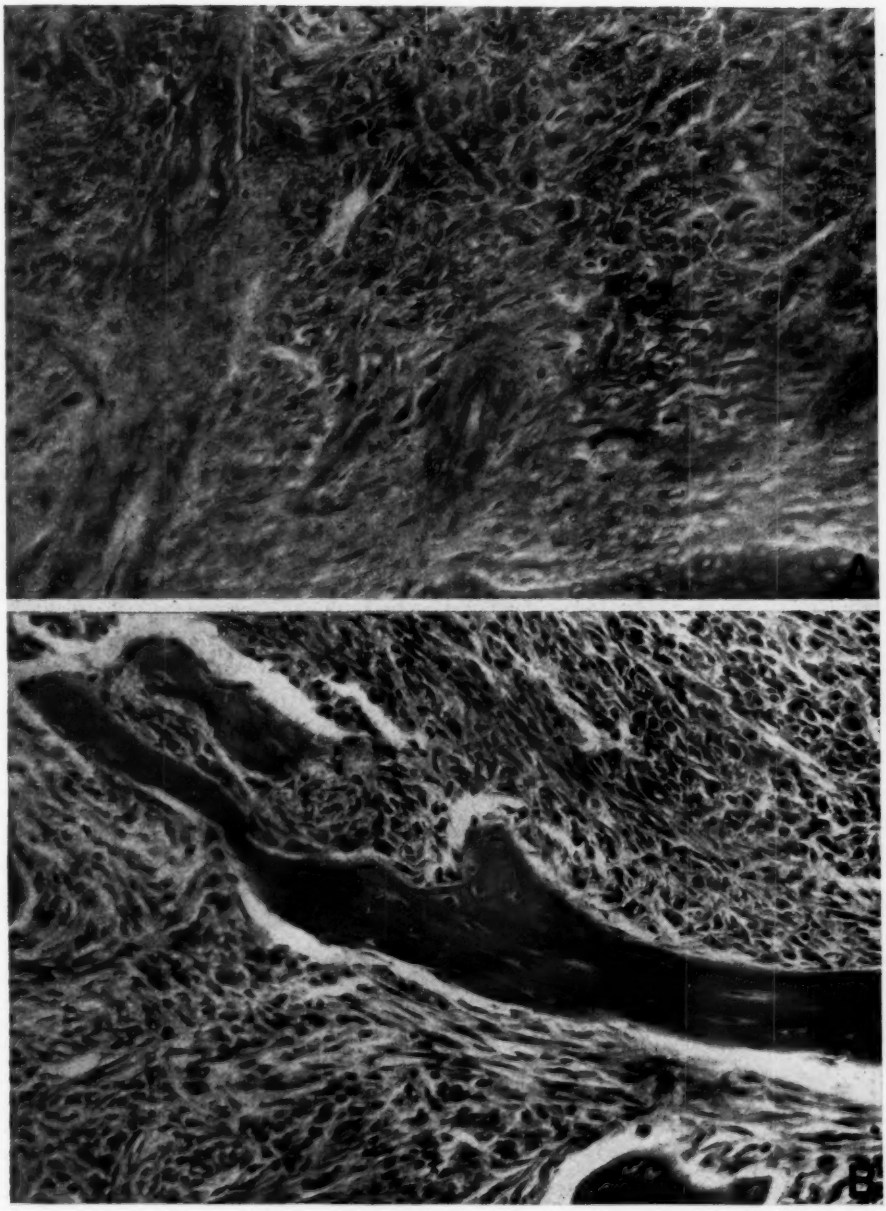

FIGURE 4.—*A*, Section from N strain tumor showing invasion of the skin by the sarcoma. Most of the structures in the corium are involved. The sarcoma cells have grown about a hair follicle to the right and are approaching the epidermal layer at the upper left; *B*, Section from L strain tumor showing invasion of bone marrow by sarcoma, with destruction of myelopoietic tissue. This section was taken from the pelvis. No remaining myeloid cells could be found in the bone-marrow cavity which lies above the large spicule of bone. Both *A* and *B* stained with haematoxylin and eosin. × 310.

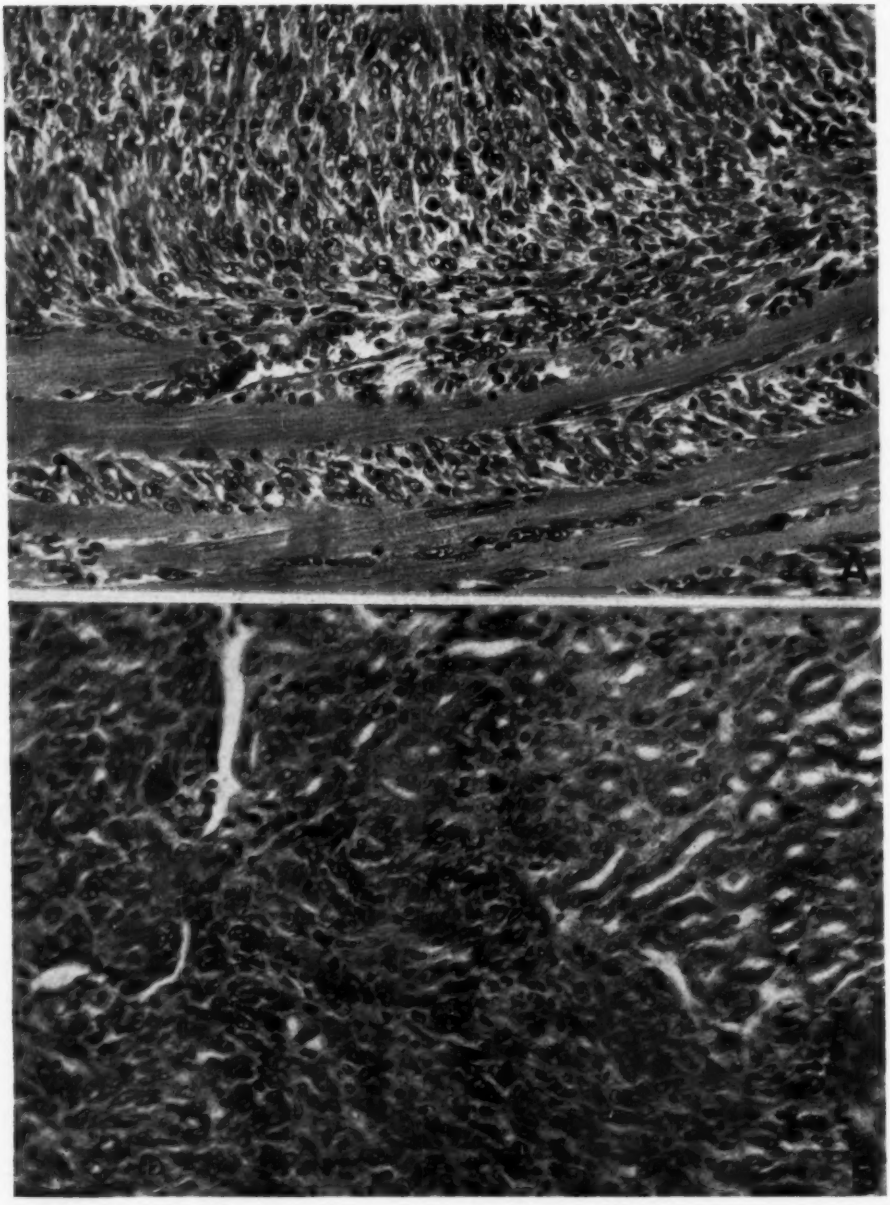

FIGURE 5.—*A*, Section from L strain tumor showing how the muscle fibers become atrophic and are finally destroyed; *B*, Almost complete destruction of the kidney structure. Some tubules are still seen at the right. Both *A* and *B* stained with haematoxylin and eosin. × 310.

being replaced by the tumor (fig. 5, B). When the invading cells reached the ureter first a hydronephrotic cortex resulted. The intestines were invaded from the serosa inward to and through the mucosa, pushing the glands aside and destroying them (fig. 6, *A*). The pancreas was in some cases almost totally destroyed, in others separated fragments of acini or islets remained, and the sarcoma cells had approached through the interstices of the pancreas (fig. 6, *B*). The splenic capsule was invaded and in one area destroyed (fig. 7, *A*). No metastases were ever found in lymph nodes, and aside from hyperplastic changes even the regional lymph nodes lying directly beside the tumors were not invaded. Figure 7, *B*, shows the single exception, a node that was almost destroyed by direct extension of the tumor.

Microscopic Studies of Metastases

In structure the metastases did not differ fundamentally from the tumor of origin except for the lack of necrosis and hemorrhage. The solitary questionable liver metastasis encountered lay directly beneath the capsule in a circular area and had destroyed all liver tissue in the area. The lung metastases were of vascular origin. One large vessel filled with tumor thrombus was found in the lung. Figure 8, *A*, shows clearly the way the sarcoma cells grew out from the walls of the vessel in a circular fashion and invaded and destroyed surrounding alveoli. The pleura was invaded, but the sarcoma had not extended beyond it. It was possible to confirm microscopically that in the cases showing metastases the tumor of origin was highly necrotic.

Necrosis was of common occurrence in all tumors beyond the first and second transplants. Even this widespread necrosis within a tumor produced very little cellular exudate. Along the edge of some of the sarcomas was a band of pyknotic polymorphonuclear leukocytes. Necrosis must occur slowly in these tumors, for even in those with the most recent areas of necrosis chronic and partially regrown areas were found (fig. 8, *B*). At most sites the remnants of necrotic cells were marked by pyknotic and karyorrhectic tumor cell nuclei and small scattered groups of leukocytes. Fresh hemorrhage was sometimes found. However, more often older areas of hemorrhage were marked by great masses of large macrophages laden with golden brown granular pigments. Growing into these areas was abundant, loose, granulation tissue, which was often mistaken, grossly, for hemorrhages. Small bands of hemorrhage lay between the granulation tissue and the living sarcoma cells. In still other areas fibroblasts and adult fibrous connective tissue were found, forming small, well-defined scar areas. Edema of both tumor and granulation tissue was common. It was observed that the more fasciculated portions of the sarcoma almost invariably lay next to granulation tissue. This suggested that fasciculation resulted when sarcoma grew out into new granulation tissue.

Variability of Cytologic Pattern

As mentioned previously, the greatest degree of variation of cytologic pattern was found. While this variation could in part be correlated with the length of time the parent tissue culture had been exposed to methylcholanthrene, nevertheless there was considerable variation within each series. In spite of the fact that the cells used were originally of the same tissue-fibroblast-culture origin, there was a distinct variation of cell pattern in the tumors. The following patterns were seen: round cell (fig. 9, *A*), angiomatous (fig. 9, *B*), neurogenic type (fig. 10, *A*), giant-cell type (fig. 10, *B*), and myxomatous (fig. 11,

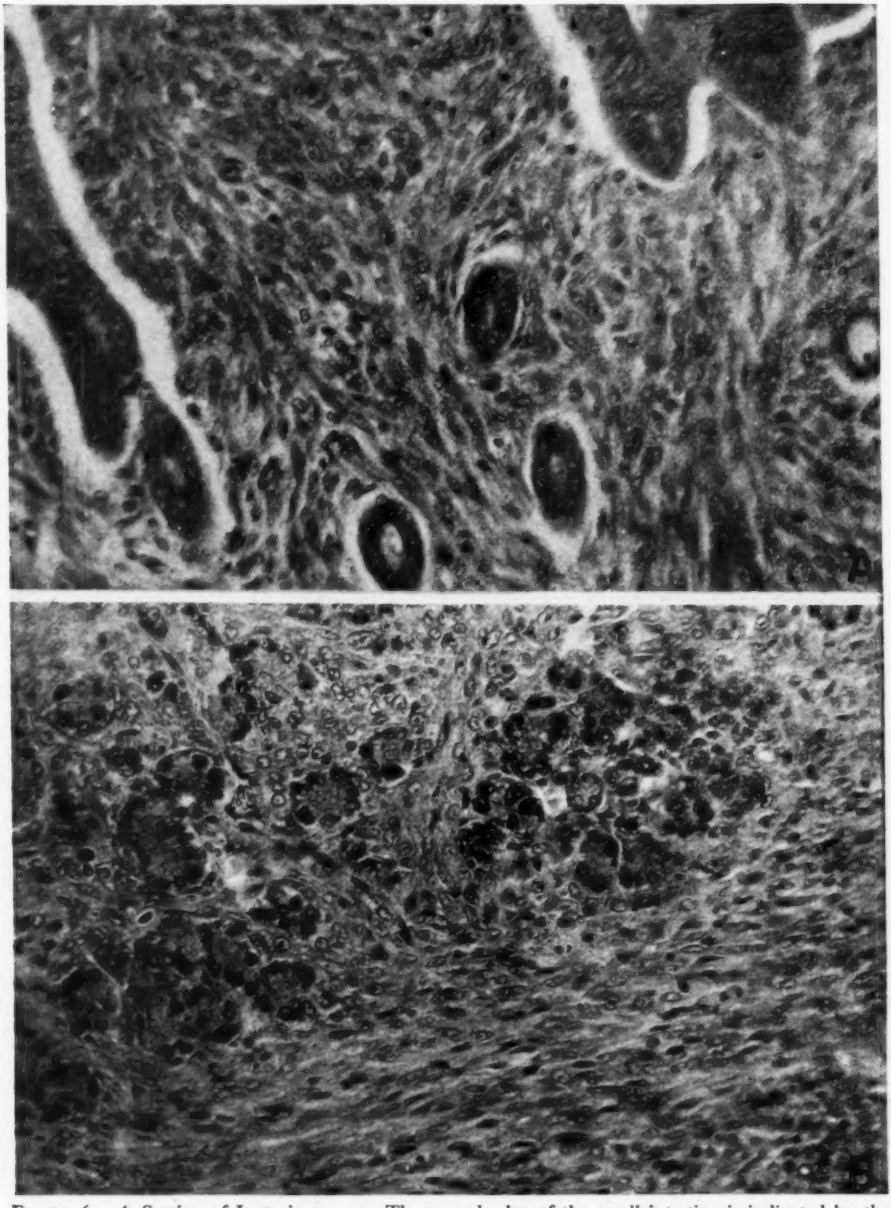

FIGURE 6.—*A*, Section of L strain tumor. The serosal edge of the small intestine is indicated by the lower border of figure. The sarcoma cells had not broken through the final layer of gland cells into the lumen in this section, but in others they had; *B*, Section from same tumor as in *A*, showing what remained of pancreatic acini. The tumor had also involved the intestine and serosal coat of the stomach. Both *A* and *B* stained with haematoxylin and eosin. × 310.

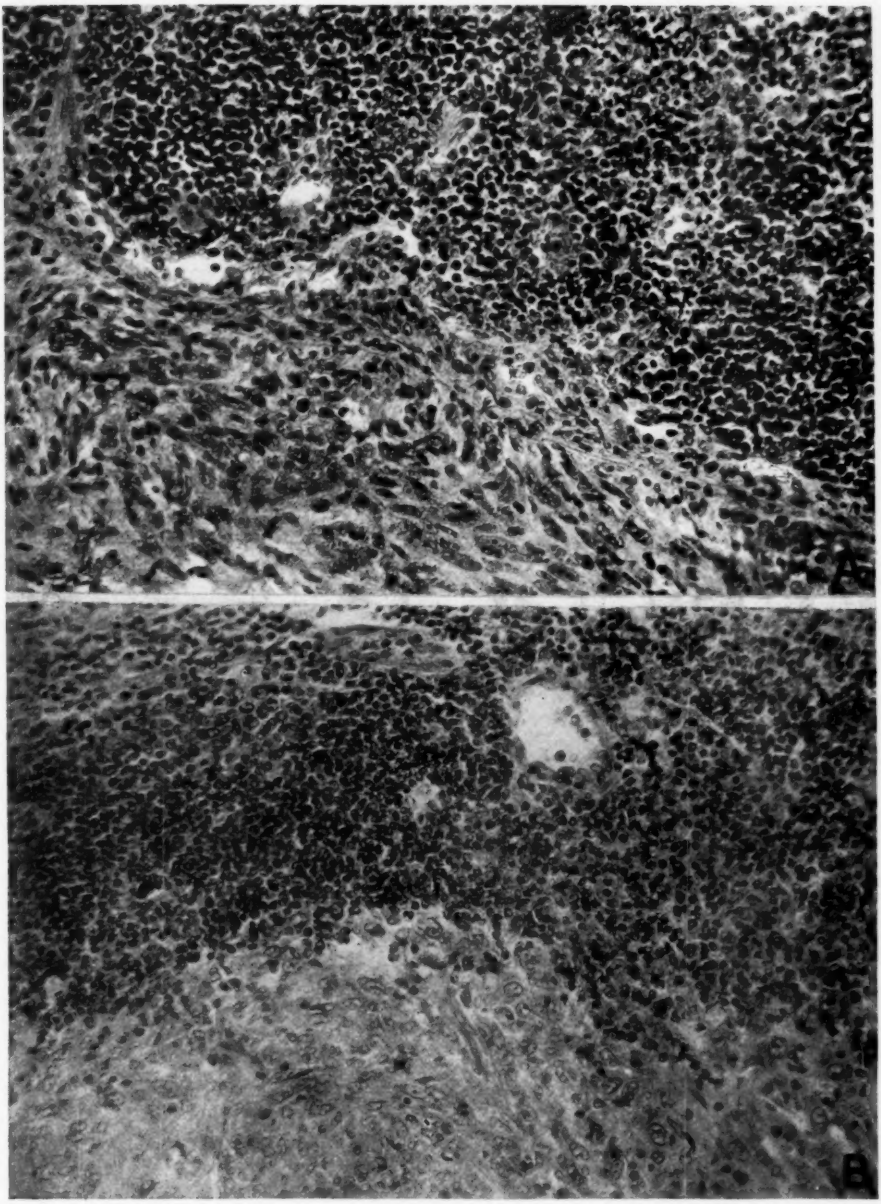

FIGURE 7.—*A*, Section from same animal as that shown in figure 6. A faint line indicates what remains of the splenic capsule; *B*, Another strain L tumor. The rim of lymphocytes is all that remains of a regional lymph node invaded by the sarcoma. Both *A* and *B* stained with haematoxylin and eosin. × 310.

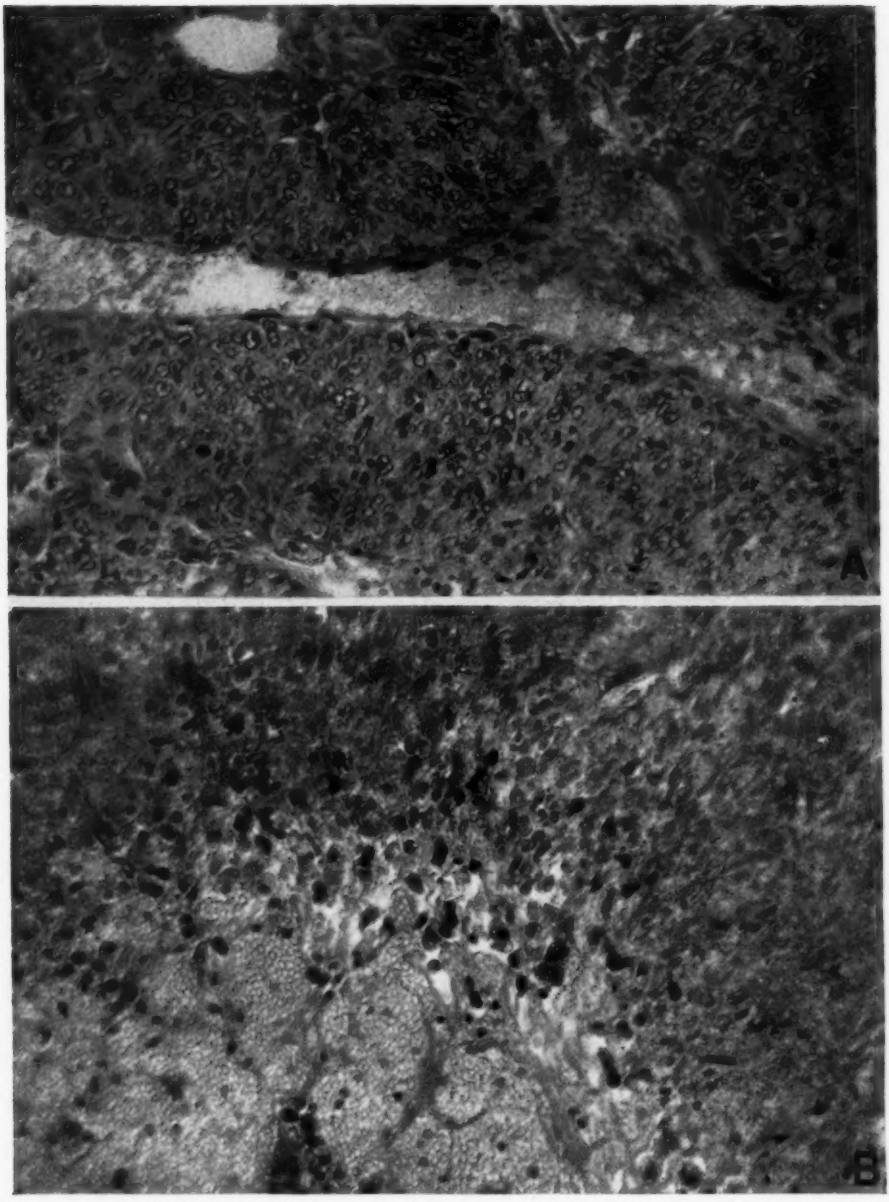

FIGURE 8.—*A*, Metastasis to the lung, of an O strain tumor. This illustrates the growth of sarcoma from an artery in the lung. The only alveoli to be seen are the atelectatic ones in the lower left-hand corner; *B*, Section from O strain tumor showing the edge of a necrotic area into which granulation tissue has grown. The gray masses of cells at the top are macrophages filled with pigment. Both *A* and *B* stained with haematoxylin and eosin. × 310.

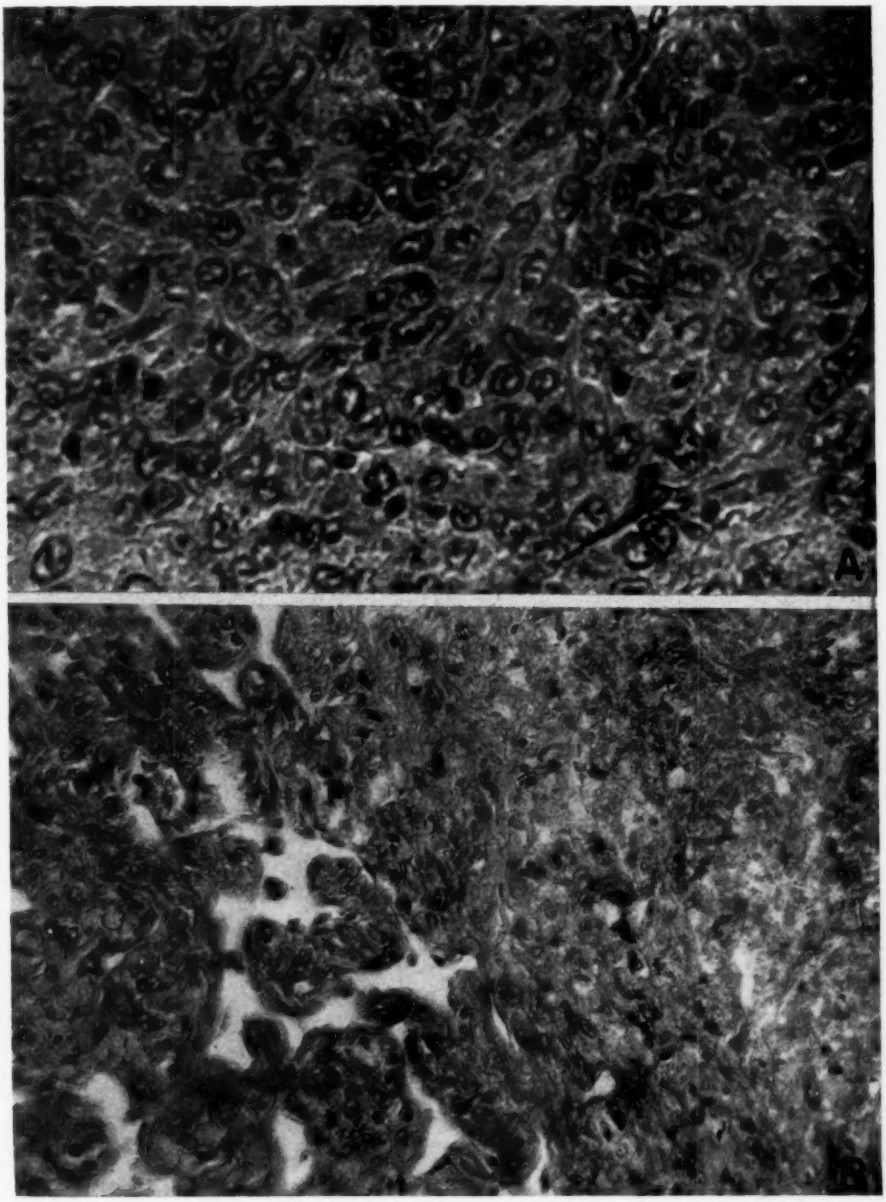

FIGURE 9.—*A*, Section from N strain tumor showing round-cell type. × 1600; *B*, Section from H strain tumor showing angiomatous type. × 310. Both *A* and *B* stained with haematoxylin and eosin.

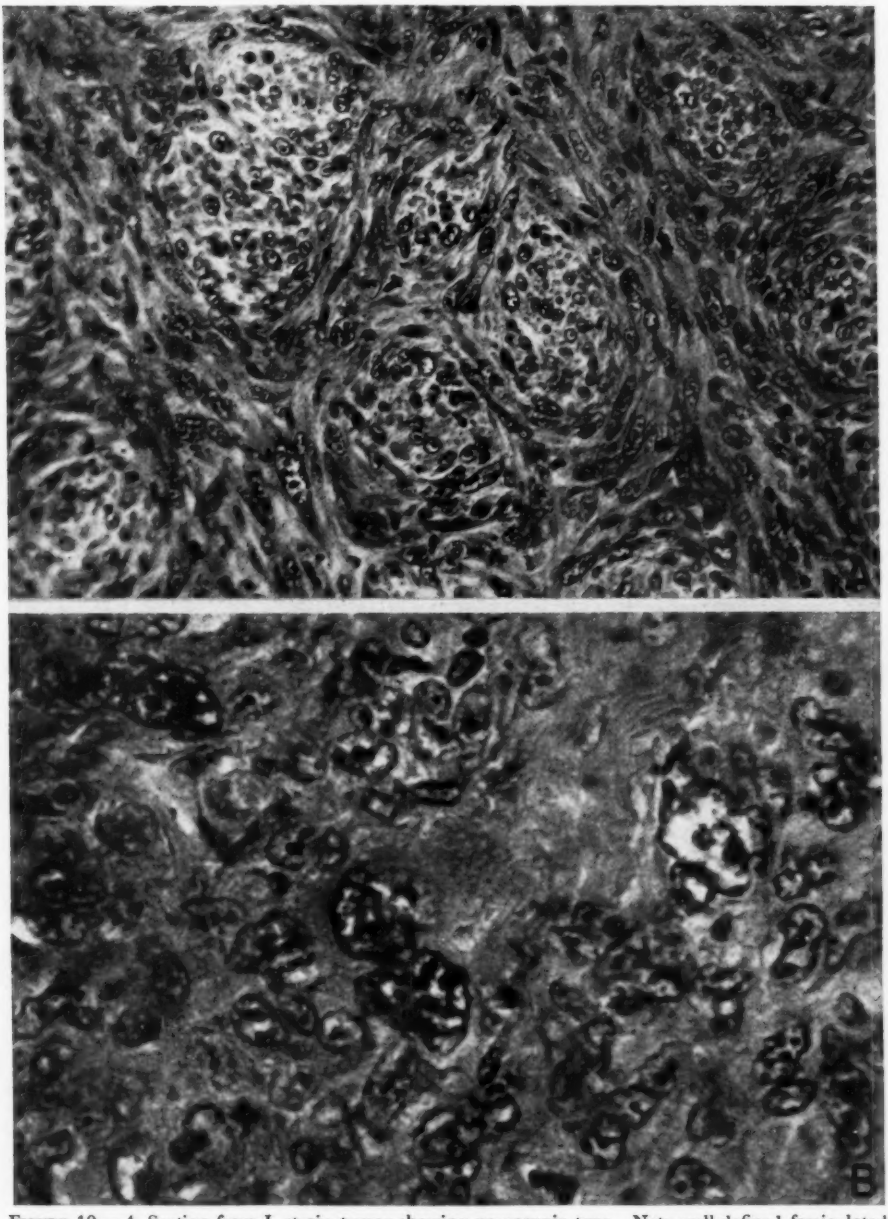

FIGURE 10.—*A*, Section from L strain tumor showing neurogenic type. Note well-defined fasciculated structure. × 310; *B*, Section from O strain tumor showing large giant cells with large nuclei. Marked irregularity in cell pattern, similar to giant-cell tumors. × 1600. Both *A* and *B* stained with haematoxylin and eosin.

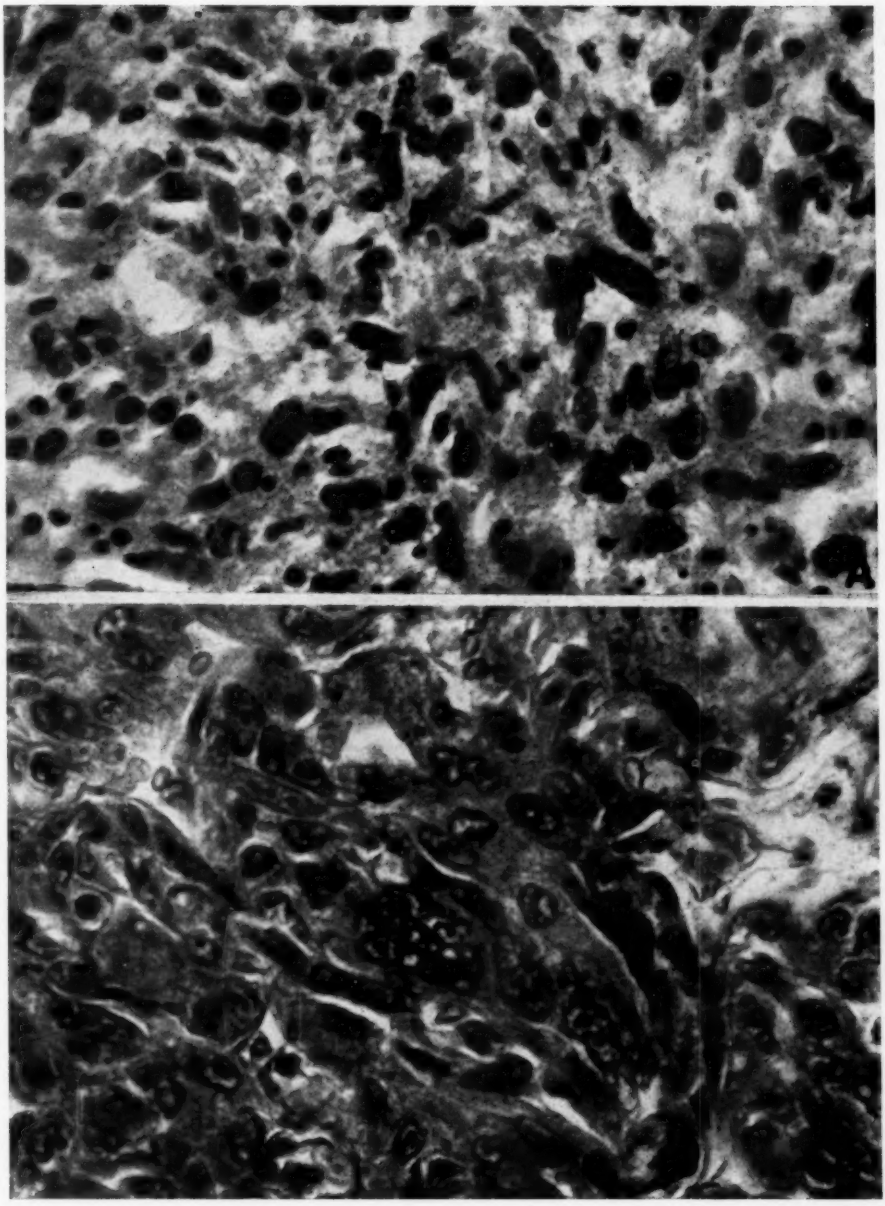

FIGURE 11.—*A*, Section from L strain tumor, myxomatous type. Marked variability in cell size, with abundant, loose, edematous, intercellular tissue. *B*. Section from O strain tumor showing a type of giant cell with multinucleation without much nuclear enlargement. Both *A* and *B* stained with haematoxylin and eosin. × 1600.

548963—43——8

A). A detailed consideration of each strain, D (control) through H, J, L, N, and O will clarify this, and it will also bring out the cell changes that may safely be attributed to the methylcholanthrene treatment of the original cultures.

Histopathology of the Various Strains

To fashion a comparative histologic basis, the following microscopic characteristics were studied and compared within each strain and from strain to strain: (1) The variability of the cell pattern, whether the cells were large or small, how they were placed, whether loosely or tightly, and the constancy of their arrangement; (2) the capacity of the cells to invade; (3) the amount of necrosis; (4) the number of mitoses (estimated per high-power field); (5) the number of giant and aberrant mitoses; (6) the occurrence of multinucleate cells and giant cells with enlarged nuclei; and (7) metastases.

Strain D

Strain D cultures were originally carried as controls. The possible explanations for their growth behavior are considered in the preceding papers (*1*, *2*). Microscopically, the tumor induced with this strain compares most closely with that of the H and J strains. Of all strains studied, strain D tumor had the most even and consistent biologic pattern. Invasion of muscle fibers by cells was found but was minimal. There was minimal necrosis. Tumors induced by this strain had fewer mitoses than those of the H strain. No metastases were found, and significantly, no multinucleated giant cells or large, single nucleated giant cells, and no aberrant mitotic configurations. Fasciculated structures were at a minimum.

Strain H

In the H strain tumor, mitoses were numerous. Invasion was present, but some of the deep injections showed a definite tendency of this tumor, unlike those seen later in the L, N, and O strains, to grow about an organ or structure rather than to invade it directly. Necrosis was about the same in quantity as in the D strain tumor, but for the first time a single case showed very few, poorly formed, multinucleated giant cells, which possessed nuclei normal in appearance. In this strain no greatly enlarged nuclei or aberrant mitoses are found, nor have any metastases appeared. There is a suggestion of somewhat greater variability in the cell pattern than in the D strain.

Strain J

The J strain tumor differs only slightly from that of the H strain, with somewhat more abundant necrosis and a slight increase in the occurrence of fasciculation of cell groups. Numerous mitoses were observed. No metastases were found.

Stratn L

Strain L is interesting from the standpoint of the first occurrence of metastases and a more malignant alteration of the sarcoma cells. The ability of the sarcoma cells in strain L tumors to invade was markedly greater than in any of the strains previously considered. The musculature, bone, kidney, and intestinal tract were invaded indiscriminately. In one case the pancreas was almost destroyed, and in another the capsule of the spleen had been invaded. Three certain cases of lung metastases occurred, with the possibility of a fourth to the lung and mediastinum. There was some uncertainty about this last case because of the possibility of direct

mediastinal extension from a heavily invaded peritoneum. Numerous mitoses were observed.

Typical case report, C–511.—Grossly, the largest mass was found to occupy the site of the left kidney and the greater part of the left side of the abdomen. It was very firm, nodular, and gray-white in color. There were multiple accessory masses scattered throughout the abdomen; one at the root of the mesentery had grown to the posterior wall of the stomach and involved the pancreas and spleen; one small mass was at the lower pole of the right kidney; and a small tumor 2 mm. in diameter was found in the lower lobe of the left lung. The microscopic examination showed that the fibrosaroma had invaded widely throughout the abdomen and was metastic to the left lung.

There was a little more necrosis than in previous strains, and mitoses were abundant. The variability in cell pattern compared closely with that of the N strain. Scattered giant cells with large nuclei were found.

Strain N

In contrast with the tumors of the previous strains, the strain N tumors began to show considerable irregularity in cell pattern. Necrosis was variable in appearance; and many tumors were free of it while others were found to be almost totally necrotic. Large giant cells with large nuclei were found more often than in the L strain. No metastases were found. Many tumors in this group were of the small-cell type. Numerous mitoses were observed.

Strain O

The O strain tumor showed a most radical departure in architecture from the tumors of the strains previously described. It presented an unusual picture, with large areas of necrosis mixed with numbers of multinucleated giant cells (fig. 12), all kinds of aberrant and bizarre mitoses, and great variability in cell pattern. Invasion was present, and with one exception no metastases were found. In this one case multiple metastases occurred.

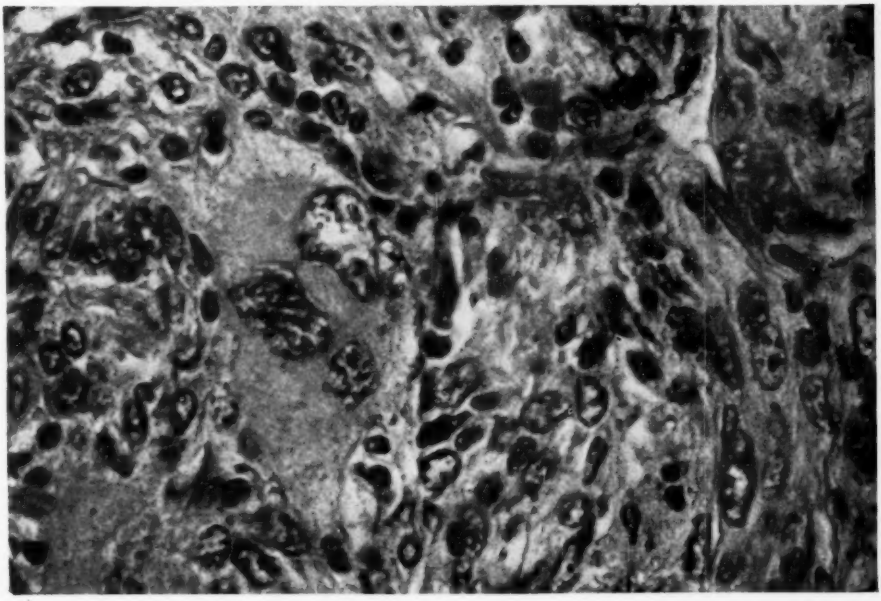

FIGURE 12.—Section of strain O tumor showing the type cytologic picture commonly encountered in this strain. Haematoxylin and eosin. × 1600.

Autopsy report, C–3470.—The original O strain tumor was situated about the left thigh. It measured 37 x 35 x 23 mm., was very soft, and on section showed widespread necrosis. The pelvis appeared to be invaded although no abdominal masses were found. The spleen was visibly enlarged. The cisterna chyli was thickened and filled with milk-white fluid. A chylous ascites was present. No obstruction of the duct could be found. The lungs showed a number of gray nodules, 2 x 2 x 3 mm., lying chiefly beneath the pleura of the left lung, but also in the right lung. A gross diagnosis of sarcoma with possible lung metastases was made. Microscopic examination revealed an invasive, highly necrotic sarcoma, with calcification, bone formation, many giant cells, and multiple metastases to the lung.

DISCUSSION

Summarizing the microscopic picture as seen in these autopsies, we have a group of animals in which the general histologic picture was essentially normal except for the tumor and its effects. In a number of animals follicular hyperplasia of the spleen was found, and the lymph node in the tumor region was often so changed. Other incidental lesions found were common to young animals. In the tumors themselves, the histologic characteristics were essentially those of a sarcoma whose morphology was altered by two factors: (1) The length of time the original culture was in contact with the methylcholanthrene was associated with a change in the cell pattern; and (2) the necrosis tended to be increased after the second tumor subinoculation.

Of these two factors, the former was more important in changing the cytologic pattern. The length of time that the carcinogen acted on the cells was correlated with the morphologic picture of the treated cells, as described in the preceding papers (*1, 2*). It also determined the pattern and behavior of the tumors that resulted from the injection of these cells. Thus, at low time-concentration doses invasive capacity was limited, mitoses were numerous but normal, and metastases did not occur. There was little variation in cell pattern, and there were no giant cells. With greater carcinogen dosage (as shown in strain L), there were numerous giant cells, an increased variability of cell pattern, increased invasiveness by the tumors, and the occurrence of metastases. With extreme carcinogen dosage (as, for instance, in tumors arising from strains N and O cells), there was an even greater variability of cell pattern. Giant cells became much more numerous, and abnormal nuclei and aberrant mitoses were common. Necrosis was increased, and there was a marked reduction in the number of metastases.

Why such a reduction of metastases should occur in the most advanced stages of carcinogen treatment is not clear because the primary tumors of strains N and O grew at such a rate that mice carrying the tumors lived at least as long as mice carrying the L strain tumors which showed such frequent metastases. If rapidity of growth of the primary tumor were the controlling influence, the H strain tumors certainly increased in size more rapidly than did the L strain, yet they showed no metastases. If, however, it can be confirmed in further work that there is such a regular reduction in the number of metastases from the tumors, the cells of which are morphologically the most greatly altered, the finding must be taken into serious consideration in predicting the clinical behavior of a neoplasm.

The large group of tumors seen grossly (578) and microscopically (285) is consistent in behavior and morphologic attributes. The tumors fall into the well-defined category of sarcomas, the origin of which is connective tissue. Their structure is similar to those known to have a spontaneous origin (*3, 4, 5, 6*), and to those induced by direct injection of a carcinogen into the subcutaneous area. They are

also similar in most respects to the sarcomas encountered in man. The great majority of them are of the large spindle-cell variety, although variants of small spindle cells are common. The large numbers of unusual cell forms in tumors produced by the injection of cultures treated longest with methylcholanthrene may be found in sarcomas but are not usual. Additional evidence derived from special stains points unmistakably to their sarcomatous nature. The wide variability in all tumor types, even though they all arose from a single tissue culture of fibroblasts, emphasizes that our ideas concerning special morphologic characteristics supposed to distinguish various types of sarcomas in man may need revision.

For comparison, there was a final small group of C3H mice which received subcutaneous injections of methylcholanthrene. The sarcomas thus produced were similar to the tumors obtained by inoculation of the tissue cultures of the various strains, except that their structure was of a small-cell type. This group most closely resembled the small-cell sarcomas from the N strain.

SUMMARY

Sarcomas were produced in normal young C3H mice by the injection of mouse fibroblast cultures which had been treated with 20-methylcholanthrene. These sarcomas were characterized grossly and histologically and found to be similar to sarcomas previously reported as having arisen spontaneously or as having been induced with carcinogens in mice and other species of animals. The neoplasms had the ability to invade surrounding structures and produce metastases; in advanced cases death of the animal resulted.

The histologic pattern of the tumors became more irregular the longer the original tissue culture remained in contact with the carcinogen. Tumors produced by cultures which had only a very short exposure to carcinogen showed more limited invasive capacity, numerous mitoses, slight variability of cell pattern, no giant cells, and no appreciable number of abnormal mitoses, nor were metastases produced.

Tumors from cells subjected to carcinogen for a longer interval (strain L) showed increased invasiveness, increased variability of cell pattern, the presence of giant cells and some abnormal mitoses, and the occurrence of a number of metastases.

Tumors from cells subjected for an even longer time showed even greater variability of cell pattern, numerous giant cells, and great numbers of distorted nuclei and abnormal mitoses. Only one instance of metastasis was recorded. Necrosis was increased over that in tumors arising from cells exposed to carcinogen for short intervals.

Five cases of metastases occurred from tumors produced by injection of cells subjected to carcinogen 111 days (strain L), whereas only a single case of metastases was found from tumors produced by injection of cells subjected to carcinogen for 184 to 406 days.

The wide variation in tumor types originating from one original tissue culture of fibroblasts indicates that our ideas of the special morphologic characteristics distinguishing various types of human sarcoma may need revision.

REFERENCES

(*1*) EARLE, W. R.: Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst., 4: 165–212 (1943).

(*2*) EARLE, W. R., and NETTLESHIP, A.: Production of malignancy in vitro. V. Results of injections of cultures into mice. J. Nat. Cancer Inst., 4: 213–227 (1943).

(*3*) HAALAND, M.: Spontaneous tumours in mice. 4th Scient. Rep. (Gt. Brit.) Imp. Cancer Research Fund, pp. 1–113 (1911).

(*4*) SLYE, M., HOLMES, H, F., and WELLS, H. G.: Primary spontaneous sarcoma in mice. Eighth communication. J. Cancer Research, 2: 1–28 (1917).

(*5*) HEIDENHAIN, L.· Über das Problem der bosärtigen Geschwülste, Eine experimentelle und theoretische Untersuchung. 153 pp. J. Springer, Berlin (1928).

(*6*) EDWARDS, J. E., DALTON, A. J., WHITE, J., WHITE, T. N.: A spontaneous fibrosarcoma of the foreleg and paw in a C3H mouse. J. Nat. Cancer Inst., 3: 191–198 (1942).